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Potassium Uptake of Normal and Low Potassium Human Red Corpuscles* (17945)

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Experiments with radioactive potassium (K^{42}) have demonstrated the continuous exchange of this ion between the intra- and the extracellular phase of most tissues including the red cells of the blood(1). Within the limits of this exchange a considerable net transport of potassium across the membrane of the erythrocytes would be possible. A net transport of potassium into the red blood cells is most likely to occur in erythrocytes with abnormally low potassium concentration. Low potassium concentration of the erythrocytes is an unusual finding even in potassium deficiency but was encountered by the authors in a case of extreme persistent potassium depletion. A quantitative estimation of the exchange of potassium in normal cells and in potassium deficient cells and an evaluation of the rate of potassium net transport into potassium deficient cells would be of great clinical interest providing a measure of the optimal rate of therapeutic supply of potassium to patients with an intracellular potassium deficiency.

In the present study such estimations were performed in *in vitro* experiments with blood samples from normal non-hypopotassemic persons and from a case of severe persisting hypopotassemia. The factors limiting the net uptake of potassium, *i.e.*, the possible exchange of Na or other cations with K moving into the cells have not been investigated in this study.

Experimental. Blood samples from non-hypopotassemic persons and from a case of severe persisting hypopotassemia were used. In the controls (Experiment A and B) the plasma potassium concentrations were 5.12

* These investigations were supported by grants from The King Christian X Funds and from Anders Hasselbalck's Fond til Leukæmiens Bekæmpelse. The radioactive K was generously supplied from the Institute of Theoretical Physics, University of Copenhagen, through Dr. Hilde Levi.

1. Hevesy, G., Radioactive Indicators, New York, 1948, 483.

TABLE I.
Potassium Uptake of Human Red Blood Cells Incubated for 6 Hours at 37°C in Plasma with Different Concentrations of Potassium. For calculations and the potassium concentrations of the erythrocytes, see text.

Exp.	Pl-K (mEq/L)	K^{42}-decrease, %	Hematocrit	K-uptake (mEq/L R.B.C.)
B	5.09	72.0	38.5	5.8
A	5.12	53.3	30.0	6.3
B	7.56	60.7	37.8	7.5
C	9.05	34.1	21.5	11.3
A	9.20	38.0	29.1	8.5
B	9.36	52.4	38.7	7.7
B	13.95	39.2	38.2	8.8

and 5.09 mEq/L and the potassium concentration of the erythrocytes 98.6 and 100.2 mEq/L respectively. The corresponding values for the hypopotassemic blood sample (Experiment C) were 1.84 mEq/L in plasma and 87.2 mEq/L in the corpuscles. Blood clotting was prevented in all samples by addition of heparin 2.5 mg per 10 ml of blood. As the hypopotassemic blood was rather anemic the controls had to be diluted with plasma from the same donors thus establishing more adequate hematocrit values. In each experiment aliquots of blood with an added amount of K^{42} were used; in some of the aliquots the plasma potassium concentration was raised by addition of KCl: in Experiment A from 5.12 to 9.2, in B from 5.09 to 7.56, 9.36 and 13.95 in separate experiments, and in C from 1.84 to 9.05 mEq/L. The entrance of potassium into the cells during 6 hours at 37°C was determined in all samples except the 1.84 specimen, which was lost. The total amounts of potassium ions having entered the red blood cells (K influx) from plasma were calculated from estimations of the plasma potassium concentration at the start of the experiment and the decrease in plasma K^{42} activity during the 6 hour period neglecting the simultaneously emigrated labelled potassium ions. By use of the relative plasma-corpuscle ratio these figures were converted into the potassium entrance into 1000 ml corpuscles. In Experiment C a net transport of potassium into the cells was calculated from a decrease in plasma potassium concentration. In the controls (Experiment A and B) no demonstrable net transport of potassium into the cells occurred.

Technic. Potassium analyses were performed in a flame photometer with a lithium internal standard. Hematocrit readings were carried out directly on capillary glass tubes at 3000 r.p.m. until constant volume; the blood column was 60 mm, bottom 170 mm from axis of the centrifuge. Each estimation is the mean of 6 readings.

Specific activity of the K^{42} used was about 1 million counts per minute per mg K. The β-emission was determined directly on samples of plasma in open special cuvettes; the layer thickness of plasma was 4 mm(2). The incubation of blood samples was performed in stoppered vials placed 11 cm from the axis of a gramophone disc rotating at 80 revolutions per minute; the disc was placed with an inclination of 45°. With this technic a gentle but thorough mixing was secured and no demonstrable hemolysis occurred. Plasma glucose and lactic acid was not measured but the pH at the end of the experiments was found to be 7.3.

Results. Potassium uptake of the red cells (mEq/L) was derived from the following figures: plasma K at the start of the experiment (Pl-K), the percentage decrease in K^{42}-activity of plasma (K^{42}-decrease) and the hematocrit (H%) using the following formula:

$$\text{K-uptake (mEq/L. R.B.C.)} = \text{Pl-K} \times \frac{K^{42}\text{-decrease}}{100} \times \frac{100-H\%}{H\%}.$$

The figures are given in Table I.

The statistical significance of the figures in

2. Davidsen, H. G., and Kjerulf-Jensen, K., *Scand. J. Clin. and Lab. Invest.* 1950, in press.

the last column (K-uptake) is sufficient in regard to the methodical error. The standard deviation of each estimation of the 6-hourly K-uptake amounts to 5% of the stated values. This figure was derived from the highest occurring standard deviation of each of the factors in the formula, *i.e.*:

Standard deviation of single plasma potassium estimations calculated from 13 tenfold estimations under identical conditions (4)	3 %
Standard deviation of estimations of K^{42}–decrease	3.5%
Standard deviation of $\frac{100-H\%}{H\%}$ (in this experiment)	1 %

The preceding figures (Table I and Fig. 1) indicate that the potassium uptake of normal red blood cells increases with increasing plasma potassium concentrations (Experiment A and B) ranging between 5.09 and 9.36 mEq/L, *i.e.* within concentrations which may be found in clinical conditions. Experiment C, however, demonstrates a potassium entrance into low potassium red blood cells, which seem to be definitely higher than the corresponding values obtained from the controls, Fig. 1. The net transport of potassium into the cells encountered in Experiment C, calculated from the decrease in plasma potassium concentration from 9.05 to 8.34 mEq/L, amounted to 3.0 mEq per liter of cells when corrected for an increase in hematocrit from 21.5 to 22.5.†

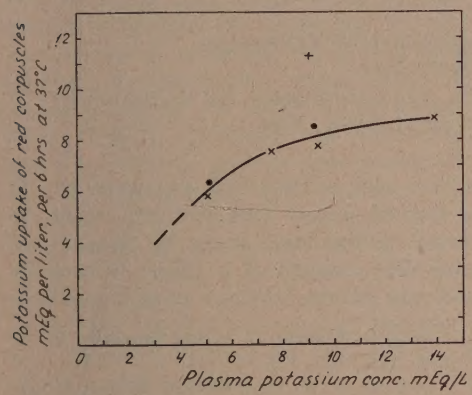

FIG. 1.

Potassium immigration into normal and low potassium human red blood cells at different plasma potassium levels. For calculation, see text and Table I.

Discussion. When potassium deficient cells are suddenly given the possibility of free potassium assimilation it would be decisively interesting to know whether the increased potassium assimilation is the result of an increased potassium uptake (K-influx) or a reduced potassium extrusion during the general exchange of ions across the cell membrane. In the preceding calculations the dilution of K^{42}-ions passing into the cells with the preexisting potassium ions of the cell interior was presumed so considerable that the number of K^{42}-ions returning to plasma was negligible. This assumption should be permissible for the following reasons: assuming a uniform exchangeability of all potassium ions in the cells and that no net transport takes place (as in Experiment A and B) a mathematical analysis reveals that only 3.7% of all emigrating ions are originating from plasma during an exchange including 7.5% of all potassium ions in the cells (the mean degree of exchange in Experiment A and B). In the case of Experiment C where in addition a net transport of potassium was demonstrated about 6% of all emigrated K-ions would be expected to be returning K^{42}-ions originating from plasma. These corrections if used in the calculations would heighten the calculated potassium uptake with 3.7 and 6% respectively. Thus the net transport in Experiment C is sufficiently explained as the result of the demonstrated increased potassium influx while the potassium emigration from the erythrocytes (K-influx — K-net transport) was quantitatively comparable with that of the controls.

The rate of penetration of labelled potas-

4. Ryssing, E., personal communication to the authors.

† $(9.05 \times \frac{100-21.5}{100} - 8.34 \times \frac{100-22.5}{100}) \times \frac{100}{21.5} = 3.02.$

sium into the red blood cells of human beings is relatively low compared with that of other tissue cells(3). Provided a total body cell K/red cell K ratio of 15 the calculated minimal 24 hour total cellular potassium uptake would be about 14 g (358 mEq) K. Actually this patient in a well controlled period prior to the experiment received 23 g (588 mEq) K during 2 days; within this period the potassium concentration of plasma increased from 2.28 to 3.45 mEq/L and of the red blood corpuscles from 94.4-99.8 mEq/L. The potassium concentration of the muscular tissue was extremely low at the time of the experiment: 50.9 mEq/kg; Na:107 mEq/kg.

Summary. The potassium exchange of normal human erythrocytes *in vitro* increased with increasing plasma potassium concentration and approached a constant level at concentrations above 9 mEq/L.

In an experiment with low potassium erythrocytes from a case of persistent hypopotassemia in which the plasma potassium concentration was raised the potassium uptake (K-influx) was found to be increased by 40% of the corresponding values for normal erythrocytes. This increase in the K-influx corresponded quantitatively to a demonstrated net transport of potassium into the low potassium red blood cells. The simultaneous emigration of K-ions, however, was not reduced, and therefore the net transport of potassium could not be considered a result of a reduced potassium emigration from the cells.

The low potassium concentrations of the erythrocytes in this particular case seemed to reflect the potassium depletion of the body cells.

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3. Moore, F. D., *J.A.M.A.*, 1949, v141, 646

In Vitro Sensitivity of Bacteria to Sulfonamide Combinations as Compared to Single Sulfonamides.* (17946)

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The therapeutic advantages of a mixture of several sulfonamides over a single effective sulfonamide are said to be due to the following: (1) A given volume of fluid (*e.g.* urine) is capable of retaining several sulfonamides in solution up to their individual maximum solubilities, so that larger doses of a mixture than the maximum for any one sulfonamide may be given without producing crystalluria. (2) On a weight for weight basis, such mixtures in a large series of cases have been given with a markedly reduced incidence of renal toxicity(1-9) and of sensitization reactions such as drug rash and fever(7). It is claimed further that mixtures are as effective antibacterial agents *in vitro* as an equivalent amount of a single sulfonamide and may in some instances exert a potentiat-

* Acknowledgment is due to Annette Freedman and Elsie Sylvester for technical assistance.

1. Lehr, D., *Brit. Med. Jour.*, 1947, vII, 943.
2. Lehr, D., *J. Urol.*, 1946, v55, 548.
3. Lehr, D., Slobody, L. B., and Greenberg, W. B., *J. Ped.*, 1946, v29, 275.
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5. Flippin, H. F., and Reinhold, J. G., *Ann. Int. Med.*, 1946, v25, 433.
6. Ledbetter, J. H., and Cronheim, G. F., *Am. J. Med. Sci.*, 1948, v216, 27.
7. Lehr, D., *Brit. Med. J.*, 1948, vII, 543.
8. Lehr, D., PROC. SOC. EXP. BIOL. AND MED., 1945, v58, 11.
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ing effect(8,9). While the reported clinical results with mixtures appear satisfactory, the clinical and laboratory data demonstrating that mixtures on a weight for weight basis possess equal or greater antibacterial potency than single effective sulfonamides is meagre or altogether lacking. Since the degree of sensitivity *in vitro* of a given strain of bacteria to single sulfonamides is a useful index of the clinical response(10), similar data may be expected to hold true for sulfonamide combinations as well. This communication presents data on the comparative bactericidal effects of a given dose of a mixture and of its individual components.

Material and methods. The *in vitro* sensitivity of bacteria to sulfonamides in culture media varies with the inhibiting properties of certain constituents in the media. To obtain the absolute range of antibacterial concentrations of a sulfonamide, it is necessary to prepare culture media completely free of inhibitors. White *et al.*(11), Kohn and Harris(12,13) and others have made use of such media. They showed that while inhibitors lower the absolute antibacterial power of sulfonamides, they do not affect their relative potency on the same strain. Since the preparation of inhibitor-free media has not been standardized or made practicable for clinical purposes, we developed a simple technic for testing sensitivity(10) which utilizes peptone broth (Difco). Although the large quantity of inhibitors in peptone broth necessitates the use of bactericidal concentrations of sulfonamides up to 250 mg % to indicate clinical effectiveness(10), the method provides a valid index of the most appropriate sulfonamide for clinical use in a given case. In the table below, the effective bactericidal concentrations should be interpreted with the above considerations in mind.

10. Schweinburg, F. B., Rutenburg, A. M., *J. Lab. and Clin. Med.*, 1949, v34, 457.

11. White, H. J., Litchfield, J. F., Jr., and Marshall, E. K., Jr., *J. Pharmacol. and Exp. Therapy*, 1941, v73, 104.

12. Kohn, H. I., Harris, J. S., *J. Pharm. and Exp. Therapy*, 1941, v73, 343.

13. Kohn, H. I., Harris, J. S., *J. Pharm. and Exp. Therapy*, 1943, v77, 1.

I. *Micro-organisms.* Seventy bacterial strains† were studied *in vitro*. Twenty-six were gram-positive cocci, as follows: hemolytic *Staphylococcus aureus* 6; non-hemolytic *Staphylococcus aureus* 4; hemolytic streptococci 8; non-hemolytic streptococci 8. Forty-four were gram-negative strains, as follows: *Escherichia coli* 6, *Aerobacter aerogenes* 6, *B. proteus vulgaris* 6, *Pseudomonas aeruginosa* 6, *Klebsiella Friedlander* Type A 2, and Type B 2; and 16 strains of the various Shigellae *(Shigella shigae,* 4; *Shigella dispar,* 2; *Shigella sonnei,* 2; *Shigella alkalescens,* 2; *Shigella ambigua,* 1; *Shigella paradysenteriae,* types V,W,Y,Z, and Newcastle) one strain each.

II. *Sulfonamide drugs.* The following sulfonamides were used in the *in vitro* tests: sulfadiazine (SD), sulfamerazine (SM), sulfamethazine (SMT), sulfathiazole (ST). They were used singly or combined in equal amounts by weight as follows: sulfadiazine and sulfamerazine; sulfadiazine and sulfathiazole; sulfamethazine and sulfathiazole; sulfadiazine, sulfamerazine and sulfamethazine; sulfadiazine, sulfamerazine and sulfathiazole.

III. *Technic.* 1000 mg % stock solutions of each sulfonamide in broth were prepared and brought to a pH of 8.2. Stock solutions of the sulfonamide combinations were prepared by mixing equal volumes of stock solutions of the individual sulfonamides. Serial dilutions of each of these stock solutions were prepared in concentrations of 750, 500, 333, 250, 100, 50, 25, and 10 mg % respectively. One cc of each of the 8 dilutions was placed in sterile Wassermann tubes. To each tube was added 0.1 cc of a bacterial suspension prepared by diluting a 24 hour culture so that 0.1 cc contained 500-5000 bacteria. A control tube containing broth without sulfonamide was also inoculated with 0.1 cc of the same bacterial suspension. All

† All cultures except the Shigellae were strains freshly isolated from patients. The Shigellae were old laboratory strains received from the American Type Culture Collection. A large number of the latter were tested because the sulfonamides are especially useful in the treatment of bacillary dysentery.

TABLE I.

Patterns of Response of 70 Bacterial Strains to Varying Concentrations (in mg %) of Single Sulfonamides and Sulfonamide Mixtures.

Patterns of response* Type	Strains	Drugs									Comment
		SD	SM	SMT	ST	SD+SM	SD+ST	SMT+ST	SD+SM+SMT	SD+SM+ST	
A	10	100	100	100	50	250	100	100	333	250	No additive action* in mixtures
B	11	250	250	250	500	250	333	333	250	333	Additive action in mixtures
C	14	>1000	>1000	>1000	250	>1000	500	>1000	>1000	750	Action of mixtures due exclusively to ST
D	8	>1000	>1000	>1000	100	>1000	250	250	>1000	500	Same
E	17	>1000	>1000	250	250	>1000	500	500	>1000	750	No additive action in mixtures
F	10	250	250	100	100	250	50	50	250	100	Potentiating action† in mixtures (SD+ST, SMT+ST)

* Additive action indicates that the bactericidal action of the mixture was equal to the sum of the action of its components.
† Potentiating action indicates that the bactericidal action of the mixture was greater than the sum of the action of its components.

tubes were incubated at 37°C for 24 hours (or longer in the case of streptococci). The lowest concentration of each sulfonamide or sulfonamide mixture, which completely inhibited macroscopically visible growth, was taken to be the bactericidal titer,‡ which is the only titer that may be considered a reliable guide in assessing the probable clinical effectiveness of a sulfonamide(10). All tests were run in duplicate.

Results. The results were classifiable into 6 patterns of response to varying concentrations of the individual sulfonamide (Table I). The bactericidal titers for the strains within a given type varied slightly, but not significantly, from the pattern shown in the Table. In the first type of response (A, Table I), the bactericidal titer of SD alone and of SM alone was 100 mg %. But the bactericidal titer of a mixture of equal parts of these two drugs was 250 mg %, indicating that the bactericidal action of the mixture was less than that of either drug alone. The same was true of a mixture of equal parts of SD, SM, and SMT, which required 333 mg % to produce a bactericidal effect. In the case of a mixture of SD and ST, the bactericidal titer was 100 mg % whereas for ST alone it was 50 mg %. There was no additive effect from the SD present. The same was true of a mixture of SMT and ST. Since a mixture of SD, SM, and ST required a concentration of 250 mg %, it is evident that not only was there no additive effect, but some suppression of sensitivity to ST may have been produced.

These data indicate for these 10 strains that at optimum concentration of a given effective sulfonamide, adding another or several others not only provides no advantage, but the reverse may occur, *i.e.*, the adequacy of a given concentration of a sulfonamide acting alone may be impaired by adding another. The ten strains in this group consisted of 3 gram-positive cocci (2 staphylococci, 1 beta hemolytic streptococcus) and 7 gram-negative bacilli.

‡ Subcultures did not reveal any growth from any clear tube, the sulfonamide concentration of which was taken to be the bactericidal titer.

In the Type B response, the bactericidal titer of some double or triple mixtures indicated an additive effect, since the concentration of each of the sulfonamides in the mixture, when acting alone, was not bactericidal. The effective titer was in a range which we would estimate to be clinically effective. All the strains in this group were gram-positive cocci: 4 staphylococci, 3 hemolytic and 4 non-hemolytic streptococci.

The character of the C & D types of response was a clinically effective sensitivity to one sulfonamide only, ST, and resistance to 3 others. The bactericidal effect of the sulfonamide mixtures was due exclusively to this one effective sulfonamide in the mixture in the same concentration required when it was acting alone. The 22 strains in these 2 types of response were 2 strains of staphylococci, 2 non-hemolytic streptococcus and 18 strains of gram negative bacilli, which included all species of gram-negative bacilli tested.

In Type E there was no evidence of an additive or potentiating influence in the various mixtures. All 17 strains in this class were gram-negative bacilli and were in a range indicative of clinical effectiveness to only 2 of the four sulfonamides tested.

In Type F the bacteria were weakly sensitive to some sulfonamides and very sensitive to others. There was a definite additive effect in some mixtures, (SD and SM), and a potentiating effect in others (SD and ST, SMT and ST). Most of the organisms were gram positive cocci, (2 strains of staphylococci, 4 of hemolytic and 2 of non-hemolytic streptococci, and one strain each of *E. coli* and *A. aerogenes*).

Discussion. An additive or potentiating effect of mixtures occurred in 21 of the 70 strains tested. Nineteen of the 21 were gram-positive cocci. A potentiating effect was also observed in one strain of *E. coli* and in one of *A. aerogenes*. No additive or potentiating effect occurred in 42 of the 44 strains of gram-negative bacilli tested. As already pointed out(10), variations in *in vitro* sensitivity to several sulfonamides are not large in the case of most cocci, but may be very considerable in the case of gram-negative bacilli. If the close correlation between *in vitro* sensitivity to single sulfonamides and therapeutic effect(10,14) also holds for mixtures, our data suggest that the favorable effect of mixtures would be expected in the case of the majority of coccal infections but not for most gram-negative bacillary infections. A favorable clinical effect of mixtures particularly in coccal infections has been reported by Lehr and others(3,4,6,9). But the effects of mixtures as compared to single sulfonamides awaits more searching clinical evaluation than is evident from the literature to date. Since (a), 27% of the gram-positive cocci and 95% of the gram-negative bacilli which we tested were less sensitive to mixtures than to single component sulfonamides, in what we judge to be a clinically effective range of concentration, and (b), individual strains of a given bacterial species may be less, equally, or more susceptible to mixtures than to single components of the mixture on a weight for weight basis, [see also Seneca *et al.* (11)] the choice between a mixture and a single sulfonamide requires determination in the individual case. On the evidence so far available, the superiority of a mixture cannot be assumed and is in fact unlikely in the case of most gram-negative bacillary infections.

Conclusion. The use of sulfonamide mixtures in preference to a single effective sulfonamide is justified only when *in vitro* sensitivity tests demonstrate for the components of the mixture either an additive or a potentiating effect. Such tests suggest that this is likely to be true in the majority of coccal infections, but not for gram-negative bacillary infections.

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Effect of Exercise upon Liver Following Partial Hepatectomy in Albino Rats. (17947)

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(Introduced by Paul György)

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This communication reports a study of the effect of exercise upon liver restoration in white rats following partial hepatectomy. The study was undertaken because of the uncertainty concerning the optimum time for resumption of physical activity by patients convalescing from acute hepatitis(1).

Procedure. Forty-two albino rats (Sprague-Dawley strain) averaging 170 (150-200) g were caged in groups of 3 and given access to unlimited amounts of a powdered stock diet. This ration contains 75% yellow corn, 16% linseed oil meal, 5% crude casein, 2% alfalfa, 0.5% sodium chloride, 0.5% calcium carbonate and 2% yeast. Body weight and food consumption were recorded daily for a few days before operation and throughout the experimental period. An estimated 70% of the liver was removed under light ether anesthesia by the method of Higgins(2). Great care was taken to leave a minimum of liver tissue distal to the ligature. The removed lobes were weighed after free blood was removed with a gauze sponge. The weight was multiplied by 1.441 to obtain the estimated total liver weight at the time of operation(3). Gurd, Vars and Ravdin found this factor to give a very accurate estimate of total liver weight by this technic. In 35 sacrificed animals we found a close approximation of their results. Twenty-four hours after the operation one half of the animals were exercised for 2 hours and thereafter for 2 hours twice a day. The rats walked approximately 200 meters per hour in a rotary, motor-driven cage. While the experimental animals were being exercised, food was removed from the cages of the controls. Thus both groups of animals had access to food for equal periods. On the third, fifth, seventh, tenth, twelfth, and fourteenth days after operation an equal number of control and experimental rats were exsanguinated under ether anesthesia. The livers were immediately removed, sponged dry and weighed. The per cent of restoration was expressed as the ratio of the estimated liver weight at the time of operation to the weight of the remnant at necropsy.

Results. The exercised animals consistently consumed a little less food than did the controls, and the gain in body weight was slower in the experimental group (Table I). Nevertheless liver restoration in the two groups was almost identical (Table I). By the tenth postoperative day an estimated 100% restoration had occurred in both groups. To gross inspection, the liver tissue present at autopsy appeared to be normal.

None of the animals failed to gain weight and eat well. At autopsy the only sign of reaction to the trauma of operation was fibrous encapsulation of the ligature knot.

Discussion. The data indicate that the amount of exercise to which the animals were exposed did not interfere with the rate or degree of liver restoration following partial hepatectomy. The nature of the restoration and character of the challenge are, of course, different from the situation presented by infectious hepatitis, where the lesion usually consists of diffuse cellular damage. In these experiments the factor of infection was absent.

It was somewhat surprising that the exercised animals ate less food than did the controls. In a separate experiment 21 non-operated rats were exercised in the same manner as were the operated group. The same

* Aided by a grant from Smith, Kline and French Laboratories, Philadelphia, Pa.

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2. Higgins, G. M., and Anderson, R. M., *Arch. Path.*, 1931, v12, 186.

3. Gurd, F. N., Vars, H. M., and Ravdin, I. S., *Am. J. Physiol.*, 1948, v152, 11.

TABLE I.
Effect of Exercise on Food Consumption, Body Weight, and Liver Restoration After Partial Hepatectomy in 42 Rats.

Days following operation	Food consumption per rat in g*		Avg body wt in g†		% liver restoration‡	
	Exercised	Control	Exercised	Control	Exercised	Control
0	15.0	14.7	169	172		
1	5.5	5.6	158	160		
2	10.2	10.3	160	165		
3	9.6	14.1	167 (±5.0)	170 (±4.1)	60.5 (±2.0)	67.5 (±4.0)
4	13.6	16.3	164	179		
5	16.8	17.7	165 (±3.7)	181 (±6.0)	79.0 (±3.5)	76.3 (±2.7)
6	15.4	19.3	170	184		
7	17.3	20.2	175 (±2.0)	186 (±3.1)	89.7 (±1.3)	94.3 (±1.1)
8	17.3	18.7	182	190		
9	15.9	16.7	187	195		
10	15.1	19.7	193 (±4.2)	201 (±1.7)	96.7 (±2.1)	95.7 (±1.7)
11	16.7	21.8	195	210		
12	19.2	20.0	197 (±10.0)	213 (±7.2)	110.0 (±3.0)	120.0 (±2.6)
13	18.8	22.5	205	216		
14	19.0	20.0	206 (±3.6)	220 (±5.0)	120.0 (±2.1)	122.0 (±1.6)

* The average difference in mean food consumption of the 2 groups was 2.31 g. The stand. dev. of the difference between the means was 1.78.

† The figures in parentheses are the stand. dev. of the cumulative wts of the animals autopsied on the days indicated.

‡ Stand. dev. in parentheses.

TABLE II.
Effect of Exercise on Food Consumption and Body Weight of 21 Non-Operated Rats.

Days	Food consumption per rat, g*		Avg body wt, g†	
	Exercised	Controls	Exercised	Controls
1	15.4	14.2	191	187
		(Exercise begun)		
2	9.3	10.2	192	191
3	11.0	13.0	196	195
4	13.3	14.5	192	191
5	11.0	13.3	192	193
6	13.7	15.0	192	196
7	12.0	13.8	193	198
8	11.0	13.5	194	193
9	14.0	14.6	195	195
10	10.7	12.0	192	201
11	10.3	13.3	198	202
12	11.3	13.5	196	203
13	15.7	16.3	194	205
14	13.0	14.3	195 (±2.9)	207 (±4.7)
		(Exercise stopped)		
15	16.5	14.5	197	208

* The average difference in mean food consumption of the 2 groups was 1.69. The stand. dev. of the difference between the means was 0.79.

† Stand. dev. for cumulative weights in parentheses.

slight but consistent decreased food consumption was apparent (Table II). The exercise appears to have been sufficiently exhausting to prevent normal eating despite the fact that exercise theoretically created a demand for additional calories. Signs of exhaustion were observed in the rats as they walked in the treadmill. The animals moved forward only when the cage rotated them to the point of discomfort.

Summary. 1. Exercise in a revolving cage did not retard liver restoration in white rats

subjected to partial hepatectomy.

2. The daily food consumption of exercised animals, both operated and non-operated, was slightly less than that of non-exercised animals.

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Chymotrypsin Inhibition by Human Serum in Health and Disease.*† (17948)

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The antiproteolytic power of blood serum in the diagnostic application to cancer has been considered(1-3). Serum also inhibits the milk clotting power of pepsin and rennin. Proteinase inhibitors may be prepared from the gastric mucosa of various animals(4), and from soy beans and lima beans as reported first by Tauber(4). Two inhibitors were recently isolated from legumes in crystalline form(5,6). The present study was well in progress when West and Hilliard(7) presented a procedure for the estimation of rennin and chymotrypsin inhibitors in blood serum, using unbuffered milk, as a diagnostic method for malignant neoplasia. In the present study strongly buffered homogenized milk of a constant pH of 5.0 is employed. This substrate produces a readily recognizable flocculant precipitate with chymotrypsin. Rennin inhibition is not included in this study.

Materials and methods. (a) *Chymotrypsin solution.* 2 mg of crystalline chymotrypsin (Worthington Biochemical Laboratory) is dissolved per cc of distilled water. (b) *Acetate buffer.* To 42 g of sodium hydroxide in 700 cc of distilled water, 115 cc of an 80% acetic acid solution is added. The mixture is cooled to 20°C and diluted to 1 liter with distilled water. (c) *Buffered milk.* Equal volumes of homogenized milk (ordinary milk may also be used but not powdered milk) and acetate buffer are mixed. The pH of the buffered milk is 5.00. (d) *Standardization of chymotrypsin solution.* All solutions are placed into a constant temperature water bath and adjusted to 20°C. 0.2 cc of distilled water and 1 mg of chymotrypsin in 0.5 cc of distilled water are placed in a 102 x 13 mm test tube. The contents of the tube are mixed. Three cc of buffered milk are added and the contents of the tube are rapidly mixed. The tube is placed in the water bath and the time necessary until a flocculent precipitate appears in the buffered milk is noted. Precipitation usually appears in 4 minutes. If the chymotrypsin solution is more or less active a new enzyme solution must be made up having a clotting time of exactly 4 minutes. The several chymotrypsin samples used in the present work appeared to be of quite constant activity. *Chymotrypsin inhibition test.* 0.2 cc of fresh serum or serum stored in the re-

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TABLE I.
Chymotrypsin Inhibitor in Patients Without Neoplasia.

	No. of cases	No. of abnormal tests	Remarks
Entire class	1556	59	
1. Pregnancy (all stages)	52	24	None of abnormal tests was below 15 wk pregnancy
2. Acute infections, active phase (bacterial and virus)	98	8	Abnormal tests included 1 epididymitis with penicillin, 1 infectious hepatitis, 4 broncho-pneumona, 1 interstitial pneumonia, 1 tonsillitis receiving penicillin
3. Cardiovascular (entire group)	41		
arteriosclerotic heart disease	12	2	
hypertension	20	3	
others	9	0	
4. Chronic infections (tuberculosis, syphilis, bronchiectasis, etc.)	193	16	Abnormal tests included 1 gangrene, 3 advanced tuberculosis, 12 penicillin treated cases (3 cellulitis, 5 syphilis, 1 necrosis of urethra, 2 abscesses, and 1 benign hypertrophy of prostate)
5. Psychoneurosis and psychosis	11	0	
6. Traumatic damage and surgical procedures	125	0	
7. Peptic ulcer, ileitis, colitis, and pancreatitis	89	2	2 abnormal tests on 1 patient who had a chronic pyloric ulcer with obstruction, and 1 with pancreatitis under penicillin treatment
8. Metabolic (diabetes, nephritis, arthritis, hyperthyroidism, and hypercholesteremia)	53	1	1 abnormal test was a diabetic receiving penicillin
9. Benign tumors (polyps, papilloma, fibromyoma, fibroma and lipoma)	90	2	2 abnormal tests on patients receiving penicillin
10. Unclassified	140	1	
11. Healthy subjects (including routine preoperative hernia, hemorrhoids, varicose veins, fractures, etc.)	674	0	Only 14 subjects showed 11½ min. clotting time. 34 showed 11 min., 78 showed 10½ min., 338 showed 8½ to 9½ min., and 82 showed 7 to 8 min.

One test was performed on each patient.

frigerator not over 24 hours is placed in a test tube using a 0.2 cc pipette. The blood need not be from fasting subjects. 1 mg of chymotrypsin in 0.5 cc of distilled water is added. The contents of the tube are mixed. 3 cc of buffered milk are added. The contents of the tube are mixed rapidly. The tube is placed in the water bath (20°C). The time necessary for the buffered milk to flocculate is noted. In the classification of the results the clotting time of 7 to 11½ minutes was chosen as the normal range (Table I, Class 11). Abnormal sera fell in the range of 12 to 40 minutes (See Tables I to III). Thus a delayed flocculation time indicates that the serum contains an increased amount of chymotrypsin-inhibitory material.

Discussion. This study includes 1556 subjects of which 60 had some type of malignant neoplasia as proved by biopsy. These patients did not receive penicillin and were free of complications such as would affect the in-

TABLE II.
Chymotrypsin Inhibitor in Untreated Patients with Neoplasia.

Diagnosis	No. of patients	Avg clotting time in min.	No. of tests performed	No. of abnormal tests
Colon (carcinoma of sigmoid); esophagus (adenocarcinoma); lung (squamous cell with metastasis); prostate (adenocarcinoma with metastasis); rectum (adenocarcinoma); skin (basal cell); stomach (generalized carcinomatosis; adenocarcinoma); lymphosarcoma; prostate (leiomyosarcoma)	14	8–11½	22	2
Cervix (carcinoma with metastasis); colon (adenocarcinoma; carcinoma of sigmoid); prostate (carcinoma with metastasis; adenocarcinoma); skin (basal cell); stomach (carcinoma); urethra (adenocarcinoma); liposarcoma (retroperitoneal); lymphosarcomatosis; testicle (teratoma with metastasis)	12	12–17½	43	29
Colon (adenocarcinoma); lung (squamous cell)	3	18–40	11	11

TABLE III.
Chymotrypsin Inhibitor in Patients with Neoplasia Under Treatment.

Diagnosis	No. of patients	Avg clotting time in min.	No. of tests performed	No. of abnormal tests
Bladder (epidermoid carcinoma); breast (adenocarcinoma); cervix (squamous cell); lip (carcinoma); lung (carcinoma with metastasis); melanoma (malignant with metastasis); rectum (carcinoma with metastasis); skin (basal; squamous cell); leukemia (chronic)	20	8–11½	47	12
Colon (carcinoma of sigmoid with metastasis); heart (adenocarcinoma); (prostate (adenocarcinoma with metastasis); rectum (squamous cell; metastatic); thyroid (adenocarcinoma with metastasis); testicle (teratoma)	10	12–17½	68	48
Bladder (adenocarcinoma)	1	18 and over	2	2

hibition of the chymotrypsin. When a large number of tests were performed on individual patients, the tests were carried out within a period of 6 to 8 months. The fluctuations of higher values to normal values were not always due to therapy. When one abnormal value was obtained, other abnormal values followed but not always in direct order. From Table I it may be seen that only 3.8% of the patients without malignancies fell in the abnormal range of which 46% were pregnancies. Only those above 15 weeks of pregnancy, however, had an abnormal anti-chymotrypsin value. 8.1% of those with acute infections and 12% of the cardiovascular disease gave abnormal tests. Those with chronic infections showed 8.2% abnormal tests which were mostly due to the patients receiving penicillin treatment. The gastrointestinal group contained 89 patients and showed 2 abnormal tests of which 1 had received penicillin. Of the unclassified group of 140 patients only 1 showed an abnormal test. Table II lists chymotrypsin values of patients with malignancies. This data shows that one single normal test is not indicative of the absence of malignancies, however, these patients were not available for further tests. In Table III

are listed chymotrypsin values of treated patients with malignancies showing that the inhibitory power of serum decreased in patients that were responsive to X-ray treatment and surgical excision.

Summary. The antichymotrypsin content of serum, as found by this test, is not changed in early malignant disease. The presence of hidden metastatic cancer, however, can be detected. Thus the test may assist in the diagnosis of advanced malignancies or in detecting such lesions when they appear in an obscure state. False positive values with this procedure fall in the same group as with the methods which measure proteolysis.

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Effect of Hypothalamic Lesions on the Peripheral Blood Picture of the Cat. (17949)

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This investigation was carried out in an attempt to verify reports in the literature to the effect that the peripheral blood picture may be influenced by the vegetative centers of the brain. The study was made using adult male and female cats which had undergone operations to produce bilateral lesions in the hypothalamus for induction of possible behavior changes. When the behavior study was completed, the animals were killed with ether, their brains fixed in formalin, embedded and sectioned for study of the areas damaged (1).

Method. The following data consist of a series of conventional blood counts and differential counts obtained from ear vein blood of 9 male and 19 female cats. The blood samples were collected for a period both before and after the production of the hypothalamic lesions. Blood counts were made with the standard pipettes and a Spencer haemacytometer. Differential counts were made after staining the smears with Wright's stain. The blood samples were taken at approximately the same time of day in each case in order to control the diurnal variations that are known to occur(2). Time of feeding was recorded in view of the possible effect it might have on the blood picture(3,4). The animals were handled as gently and quietly as possible in order to avoid emotional disturbances which also are known to have a marked effect on the peripheral blood picture(5). The number of counts made on each animal varied somewhat, ranging from 6 to 9 in the preoperative period and from 6 to 8 counts in the post-operative period.

Findings. In the normal cats the blood picture before operation showed an average WBC count of 20,017 for the males, with a range from 10,361 to 28,950. After hypothalamic damage* the average WBC count was 25,224 with counts ranging from 10,333 to 35,117. Except for one male cat which showed a .3% decrease in the average post-operative count, the remaining males showed individual increases in WBC counts ranging from .5 to 49.1% (Table I). The average WBC count before operation for the females averaged 22,711 with a range from 13,525 to 32,786. After operation the average WBC count for this group was 22,962, an increase over the average count before operation.

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* For extent and location of hypothalamic lesions see Wheatley(1).

TABLE I.
Average White Blood Cell Counts Taken on Male and Female Cats Before and After Operation to Produce Hypothalamic Lesions.

Cat	Before operation Behavior normal WBC count	After operation		% increase or decrease
		Behavior change	WBC count	
Male				
P-26	10361	None (normal)	10333	— .3
19	24019	" "	24143	+ .5
13	16279	" "	28369	+12.8
44	21014	" "	25642	+22.0
15	27375	Slight-variable	30381	+11.0
28	28950	" "	35117	+21.3
37	21867	" "	28700	+31.2
43	17379	" "	25908	+49.1
18	12911	Decided	18421	+42.7
Avg	20017		25224	
Female				
P-33	32786	None (normal)	25217	—23.1
39	19350	" "	22529	+16.4
17	24239	" "	29309	+20.9
16	29281	" "	38157	+30.3
32	17129	" "	22808	+33.2
27	25093	Slight-variable	19967	—20.4
40	28850	" "	23975	—16.9
36	24317	" "	21142	—13.1
38	26033	" "	25683	— 1.4
25	21644	Decided	18229	—15.1
41	25607	"	21800	—14.9
23	17889	"	17264	— 3.5
35	20833	"	21673	+ 4.0
24	18811	Savage	17964	— 4.5
30	17000	"	17000	—
31	18874	"	20050	+ 6.2
20	20378	"	31650	+ 6.2
29	29864	"	24258	+16.3
42	13525	"	17586	+29.3
Avg	22711		22962	

Post-operatively, the average counts ranged from 17,000 to 38,157 (Table I). Here again, as in the male series, there is an apparent slight increase in the average white count after hypothalamic damage. Nine female cats showed individual increases in average WBC counts after the operation, ranging from 4.0 to 33.2% increases. After the production of lesions 9 females showed a decrease in average WBC counts ranging from 1.4 to 23.1%, while in one female the average count was exactly the same after operation as it was for the preoperative period (Table I). It may be concluded that there is probably no significant change in the number of white cells when the range of variation is taken into consideration.

Examination of the data for the RBC count showed the group count for the males average 6,764,414 with a range from 5,661,111 to 7,898,571 in the preoperative period. After operation the average count was 7,116,580 with a range from 5,391,250 to 9,525,000. In the group of males, 2 animals showed a decrease in RBC count after operation ranging from 5.5 to 11.0% and 7 showed an increase ranging from 1.0 to 31.7% (Table II). In the female group the average RBC count before operation was 6,878,489 with a range from 4,300,000 to 8,497,143. After operation the average count dropped slightly to 6,747,970 with a range from 5,073,333 to 8,170,000. In this group 9 animals showed a decrease after operation ranging from 3.8 to 23.0% and 10 females showed an increased RBC count after hypothalamic damage that varied from .8 to 22.0% (Table II). In the RBC series, as well as in the WBC study, any

TABLE II.
Average Red Blood Cell Counts Taken on Male and Female Cats Before and After Operation to Produce Hypothalamic Lesions.

Cat	Before operation Behavior normal	After operation		
		Behavior change	RBC count	% increase or decrease
Male	RBC count			
P-26	5661111	None (normal)	5720000	+ 1.0
19	7235000	" "	9525000	+31.7
13	6060000	" "	5391250	—11.0
44	7898571	" "	7903333	+ 1.3
15	7617500	Slight-variable	8308750	+ 9.1
28	6988571	" "	7403500	+ 5.8
37	6521667	" "	6778333	+ 3.9
43	6152858	" "	5813333	— 5.5
18	6744444	Decided	7205721	+ 6.8
Avg	6764414		7116580	
Female				
P-33	5951429	None (normal)	7258333	+22.0
39	7448333	" "	5770000	—22.5
17	6394444	" "	6233333	—10.1
16	7128750	" "	7617143	+ 6.9
32	4300000	" "	5073333	+18.0
27	7617143	Slight-variable	6886667	— 9.6
40	8497143	" "	6855000	—19.3
36	6793330	" "	5775000	—15.0
38	6756667	" "	7513333	+11.2
25	6528889	Decided	7142857	+ 9.4
41	6024286	"	5795000	— 3.8
23	6617778	"	5875714	—11.2
35	7285000	"	8170000	+12.1
24	6550000	Savage	6984286	+ 6.6
30	7888571	"	7068333	—10.3
31	8227143	"	6233333	—23.0
20	6936667	"	7141429	+ 3.0
29	6415714	"	7431667	+15.8
42	7330000	"	7386667	+ .8
Avg	6878489		6747970	

attempt to correlate these doubtful changes with the degree of behavior change in the post-operative animals or with the areas or degree of damage only gave negative results.

The data on the differential counts were likewise negative and show marked individual variability. Examination of the average differential counts for the individual animals, both male and female, showed considerable variation in the post-operative responses of these animals. These changes are not constant, however, even within the various groups and they cannot be correlated with the areas damaged or the degree of damage produced.

Discussion. From study of the data of this investigation, the inconsistency and variability of the individual responses of both male and female cats to hypothalamic damage raises a question concerning the interpretation of comparable studies by previous investigators. Many investigators have reported changes in the peripheral blood picture following various experimental procedures on the central nervous system. Rosenow(6) has reported neutrophilic leucocytosis after intraventricular injection of a suspension of kaolin. Schulhof and Matthies(7) observed a polyglobulia of long duration after the injection of siliceous earth into the proximal part of the vegetative centers of the brain in rabbits. Hecht and Weil(8) found a polycythemia after cerebral trauma. Following bacterial

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8. Hecht and Weil, *Deutsche Med. Woch.*, 1929, v55, 380.

protein injection, Hoff and von Linhardt(9) reported that in man and animals there was a fever combined with a marked leucocytosis. However, after sectioning the cord, no change was observed following the bacterial protein injection, but leucocytosis could still be produced by experimental acidosis and it was concluded that peripheral changes as well as central nervous activity influence the blood composition.

Other similar results have been reported, but all are deficient in a precise delimitation of nuclei and structures of the brain involved by the lesions. Furthermore, many of the results were complicated by the presence of a foreign substance in the brain. A very comprehensive review and evaluation of the pertinent papers concerning the role of the central nervous system in affecting hematopoiesis was published in 1944 by Lucia and Marasse(10). In a summary these authors state "that the experimental evidence offered to establish the existence of an erythropoietic or a leukopoietic center in the diencephalon is insufficient." From our data it is evident that the types of hypothalamic destruction in the cats studied could not be responsible primarily for any consistent changes occurring in the peripheral blood picture in this particular group of animals. If the male and female group averages only had been considered, our findings would have been comparable to the results published by Rosenow, *et al.*(6), who reported rises in peripheral blood counts after hypothalamic damage. However, if the responses of the individual animals are considered no such consistent rises are found. One is aware only of the variability in the response of the individual animals to lesions placed in similar areas of the brain.

As noted previously, Izquierdo and Cannon (5) found that emotional disturbances have a marked effect on the peripheral blood picture. No doubt hormonal and humeral factors are the immediate effectors of the changes produced. By way of attempting to explain the extreme range and variability of the blood counts in these animals, and particularly in the animals made savage, attention should be called to the fact that these animals undoubtedly were subjected to various emotional situations beyond our control. Even with great care in handling, certain of the cats displayed signs varying from uneasiness to fright and rage. Irritated cats and those showing post-operative savagery and rage all evidenced signs of increased autonomic activity and the associated release of chemical effector substances might have affected the blood picture. Although there were outward manifestations of emotional change in our animals, we had no means of measuring the activity of the central nervous system to ascertain a possible correlation.

Conclusions. Blood counts and differential counts were taken on a series of 28 cats before and after production of hypothalamic lesions for study of behavior changes. The average blood counts for individual cats (male and female) post-operatively is marked by the variability of response. Attempts to correlate the post-operative changes with areas damaged, degree of damage, or with behavior changes gave negative results. It seems apparent that the peripheral blood picture of these animals is not primarily under the control of the hypothalamic areas damaged by these lesions, since it does not show consistent permanent changes. Nor is the blood picture affected apparently by neighboring areas whose fiber tracts traverse this region and would be interrupted by the lesions.

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Effects of Irradiation on Nucleic Acid Formation.* (17950)

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Hevesy and his associates have shown that irradiation of a tissue depresses the formation of desoxypentose nucleic acid in it(1,2,3). The method used by them consists in irradiating animals, injecting a tracer dose of $Na_2HP^{32}O_4$ and sacrificing after a given time interval. The desoxypentose nucleic acid is then isolated and the specific activity of its phosphorus is determined. This is an index of the amount of newly formed nucleic acid during the time interval under investigation. Using this technic Ahlström, Euler and Hevesy(4) also demonstrated an indirect effect of radiation probably due to some humoral agent. They used rats with bilateral transplants of Jensen sarcoma. In each animal one tumor was irradiated with 2000 r and the other tumor was shielded, yet the depression of the nucleic acid turnover rate was similar in both groups. Experiments to be presented in this paper demonstrate the presence of an indirect effect when liver or muscle rather than tumor tissue is irradiated.

Methods. Except for the method of irradiating the animals, the plan and procedures used in these experiments very closely followed those used by Hevesy and his associates. The animals used were female A strain mice bearing bilateral transplants of Strong's mammary carcinoma. They were irradiated on the tenth or eleventh day after transplanting when the average tumor weight was about 0.6 g.

Irradiation method. The easiest way to irradiate specific tissues in a large number of animals is to inject some radioactive substance which localizes in these tissues. We have found colloids containing radio-yttrium very useful for this purpose. The chemical and biological properties of these colloids have been reported: Gofman(5a), and Dobson, Gofman, Jones, Kelly, and Walker(5b). The yttrium-hydroxy-citrate developed by Gofman for liver irradiation was made in the following manner: 0.10 ml of 0.2 M yttrium nitrate solution (incorporating the desired yttrium radioisotope) is mixed with 0.18 ml of 0.05 M sodium citrate solution; 1 drop phenol red indicator is added, and the mixture is titrated to the salmon colored end point with 1 M sodium hydroxide. It is then diluted to 1.0 ml with water. The colloid used was made with Y^{90} which has a half life of 65 hours and emits only beta rays with a maximum energy of 2.2 MEV. It may be separated at frequent intervals from a Sr^{90} parent source.

When the colloid is injected intravenously into mice, 95% of it disappears from the blood stream with a half time of 88 seconds. Twelve mice killed 4 hours after the intravenous injection showed the following average distribution: Liver and spleen 84%, carcass 9%, tumors 0.95%. When the colloid was injected intramuscularly 4 hours later most of it was still at the site of injection. The fraction which had been removed from the site showed the following average distribution for 8 mice: Liver and spleen 2.1%, carcass 4%, tumors 0.2%. Our calculations based on current information on the Y^{90} β spectrum indicate that a large block of tissue in which one microcurie of Y^{90} is distributed uniformly in each gram of tissue absorbs 154 ergs per gram of tissue per hour. In the case of mouse livers one must also take into consideration the fact that the half thickness for Y^{90} beta

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4. Ahlström, L., Euler, H., and Hevesy, G., *Arkiv. Kemi, Mineral., Geol.,* 1945, 19a, No. 13.

5. (a) Gofman, J. W., *J. Lab. and Clin. Med.,* 1948, v34, 297; (b) Dobson, E. L., Gofman, J. W., Jones, H. B., Kelly, L. S., and Walker, L. A., *J. Lab. and Clin. Med.,* 1948, v34, 305.

radiation is approximately 0.1 cm in tissue and hence not all of the energy will be absorbed in the liver. Jones *et al.*(6) have calculated that for beta rays with the above half thickness only about 73% of the beta radiation originating in the liver is absorbed in that organ. The average liver weight of the mice used was 1.7 g and the average Y^{90} dose administered was 900 microcuries per mouse. During the 5 hour period of the experiment then, approximately 4.25×10^5 ergs total or 2.5×10^5 ergs per g (3000 rep‡) were absorbed by the liver. The tumors of these animals received approximately 5×10^3 ergs (50 rep). In the muscle irradiation experiments the yttrium colloid was injected into the gastrocnemius. It is estimated that approximately 90% of the beta radiation originating in the muscle was absorbed. The average Y^{90} dose administered was 630 microcuries which during the 6 hour period of the experiment delivered about 5.25×10^5 ergs to the muscle. Due to the small amount of Y^{90} which left the muscle, the livers of these animals received 5.9×10^3 ergs per g (71 rep) or 1×10^4 ergs total and the tumors received 8.5×10^2 ergs per g (10 rep) or 1×10^3 ergs total.

The whole body x-irradiation experiments were done with 180 KV X-rays filtered through 0.45 g per cm^2 of copper and 0.3 g per cm^2 of aluminum. The dose, as measured by a Victoreen meter was 60 roentgens which is equivalent to 5.6×10^3 ergs per g of tissue or 1.4×10^5 ergs total.

Plan of experiments. The procedure followed for the liver irradiation experiments was as follows: The yttrium colloid was injected into the tail veins of the mice and three hours later the tracer dose of sodium phosphate was given intraperitoneally. The mice were sacrificed exactly 2 hours after the phosphate injection. In order to obtain enough purified desoxypentose nucleic acid the tumors from four animals had to be pooled. The average tracer dose used per mouse was about 20 μc which is equivalent to 1.46×10^7 counts per minute on the counter used for the assay of the nucleic acid phosphorus. The material injected contained less than 0.01 mg Na_2HPO_4 in isotonic saline so that it did not upset the phosphate balance of the mice. In the experiments where the muscle rather than the liver was irradiated, the yttrium colloid was injected into the hind leg in the general region of the gastrocnemius. The tracer dose of phosphate was given four hours later but in all other respects the procedure was identical with that used for the liver irradiation experiments. The liver desoxypentose nucleic acid was also isolated. In the experiments with the low total body x-irradiation, approximately half the dose was given in one hour before the intraperitoneal injection of tracer phosphate and the other half in one hour immediately following it. In all other respects it was like the experiments above.

Nucleic acid isolation procedure. The method used for the isolation of the desoxypentose nucleic acid was essentially Levene's as modified by Klein and Beck(7). Some changes were necessary in order to eliminate the Y^{90} and to make the method suitable for a tracer experiment. Five to 10 g of tissue were ground with sand in mortar and pestle and mixed with 10 ml of 5% sodium chloride. The tissue was then boiled in a water bath for a few minutes, 0.25 ml glacial acetic acid added and then made basic with 0.5 g sodium hydroxide and 0.1 g sodium acetate. The basic mixture was boiled for about one hour or until the organs were almost completely dissolved. One ml of glacial acetic acid and 0.7 ml of a 5% dialyzed ferric hydroxide solution were then added. After standing a short time, another cc of acetic acid was added and the solution centrifuged. The supernatant was treated with an equal volume of methyl alcohol and the crude nucleic acid was centrifuged off. In order to purify the desoxypentose nucleic acid it was dissolved in 5 ml of 1 M sodium hydroxide and the following solutions added: 0.2 ml of a saturated solution of disodium phosphate, 0.2 ml 1 M sodium fluoride, 0.2 ml 0.2 M yttrium nitrate, and an equal volume of methyl alcohol. After heating in a water bath of 65°C

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‡ 1 rep = 83 ergs per g.

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TABLE I.
Averages of Tumor Desoxypentose Nucleic Acid Specific Activities.

	No. of animals	
Controls	320	$2.50 \pm 0.045 \times 10^{-3}$
Livers irradiated with 4.25×10^5 ergs	136	$1.65 \pm 0.052 \times 10^{-3}$
Muscles irradiated with 5.25×10^5 ergs	160	$2.10 \pm 0.042 \times 10^{-3}$
60 r total body X-irradiation equivalent to 1.4×10^5 ergs	92	$2.23 \pm 0.041 \times 10^{-3}$

TABLE II.
Averages of Liver Desoxypentose Nucleic Acid Specific Activities.

	No. of animals	
Controls	188	$3.83 \pm 0.19 \times 10^{-4}$
Muscles irradiated with 5.25×10^5 ergs	148	$2.83 \pm 0.115 \times 10^{-4}$
60 r total body X-irradiation equivalent to 1.4×10^5 ergs	84	$3.16 \pm 0.26 \times 10^{-4}$

for 15 minutes, the impurities were centrifuged off. The supernatant solution was placed in an ice bath, acidified with 3 M hydrochloric acid, and diluted with an equal volume of methyl alcohol. The nucleic acid was then centrifuged off. The purification was repeated 6 times. After this the nucleic acid was dissolved in sodium hydroxide and reprecipitated with hydrochloric acid and methyl alcohol 4 more times and finally dissolved in about 5 ml of 0.1 M sodium hydroxide.

Klein and Beck found that the nucleic acid was pure by chemical criteria after only 3 repurifications. However, as can be seen from the values given below, 8 to 10 precipitations were found necessary in this experiment in order that the specific activity of the nucleic acid remain constant upon successive reprecipitation. In a typical liver sample the following values were obtained on aliquots taken after the stated number of precipitations:

No. of precipitations	Specific activity
3	27.1×10^{-4}
6	4.9×10^{-4}
8	3.7×10^{-4}
10	3.9×10^{-4}

Determination of specific activity. The specific activity of the purified desoxypentose nucleic acid was determined in the following manner. One aliquot of the sodium nucleate solution was used for the determination of the phosphate concentration by the method of Fiske and Subbarow. A second aliquot was using for counting the P^{32}. In order to detect any possible contamination of the P^{32} with Y^{90} the samples were recounted a week later. Since the half life of Y^{90} is only 65 hours compared to the 14 day P^{32} half life, the few percent of Y^{90} occasionally still present could be eliminated. All counting was done with an accuracy of at least 2%.

Results. All the values given represent the number of P^{32} counts per milligram of phosphorus divided by the number of counts injected, normalized for the weight of the mice. Errors quoted are $1\sigma_M$.

Discussion. In this experiment no attempt has been made to measure the absolute turnover rate of the nucleic acid. Hevesy's method of calculating it from measurements of the specific activity of the nucleic acid and the specific activity of the inorganic phosphate is not justified as has been pointed out by Chaikoff(8). In order to arrive at a correct value for the turnover rate one would have to know the intermediates and rate determining steps in the nucleic acid synthesis and this is to date not possible. Hevesy and his associates have shown that the rate of entrance of radioactive phosphate into the cells of normal rat tissues or Jensen sarcoma is not affected by the radiation doses used in the present experiments. In other words the specific activity of the intracellular inorganic phosphate was the same in the control and irradiated animals. Any difference in the specific activity of the nucleic acid phos-

8. Chaikoff, I. L., *Physiol. Rev.*, 1942, v22, 291.

phorus then must have been due to a difference in the rate of incorporation of phosphate into the nucleic acid. The specific activity may be considered equivalent to the relative turnover rate.

The desoxypentose nucleic acid turnover rate as measured by P^{32} is now generally thought to be an index of cell division. Several observations strongly suggest that this nucleic acid is synthesized only during cell division and that phosphate is turned over only during this synthesis. First, the relative turnover rates for various tissues as measured by Hevesy and others closely parallel the known relative mitotic indices for these tissues. Second, Hevesy and Ottesen(9) found that nucleated hen erythrocytes (which are non-dividing cells), in contradistinction to all other tissues tested, formed no active desoxypentose nucleic acid when incubated with radioactive phosphate. Last, Spiegelman and Kamen(10) found that in labeled actively metabolizing yeast cells under conditions where there was no cell division or protein synthesis there was no change in the specific activity of the nucleoprotein phosphorus. Aside from the difficulties still present in the interpretation of nucleic acid specific activities there is the technical difficulty of measuring it. The standard methods of chemical separation are not designed for the degrees of purity necessary in some tracer experiments, particularly when the substance to be measured has a very low turnover rate as is the case with desoxypentose nucleic acid.

In the present experiment the nucleic acid was reprecipitated until the specific activity remained constant on successive precipitations. This seemed the only feasible criterion of purity. However, it is possible that some of the more active phosphate groups which were thus eliminated were actually more labile components of desoxypentose nucleic acid rather than impurities. At the present time not enough is known about the chemistry and structure of nucleic acids to settle this question.

9. Hevesy, G., and Ottesen, J., *Nature*, 1945, v156, 534.

10. Spiegelman, S., and Kamen, M. D., *Science*, 1946, v104, 581.

The experiments reported here confirm the results of Ahlström *et al.*(4) as to the existence of an indirect effect of radiation on the desoxypentose nucleic acid turnover rate. The tumors in those animals which received 4.25×10^5 ergs to the liver had 66% of the nucleic acid specific activity of the control group. By direct radiation these tumors received 5×10^3 ergs which, according to the total body x-irradiation experiment, would have depressed it to 90% without the indirect effect.

The results in the muscle irradiation experiment are not quite as clear cut. Although the total energy delivered to the muscle was somewhat greater than that delivered to the liver in the earlier experiment, the volume of tissue irradiated was much smaller, which might possibly account for the smaller effect. The tumor nucleic acid specific activity was 84% of the control value and it is felt that nearly all of this depression was due to an indirect effect. These tumors, due to the radioactive colloid contained in them, received one-fifth as much radiation as the total body x-irradiation group so that the direct radiation was negligible. Also, there was no difference between those tumors close to the irradiated muscle and those far removed, indicating that essentially none of the radiation originating in the muscle had reached the tumors.

In this experiment the liver desoxypentose nucleic acid was also measured. However, due to the colloid localized in them these livers received approximately the same dose of radiation as the total body x-ray group and the specific activities do not differ significantly.

Summary. Mice of the A strain bearing bilateral transplants of mammary carcinoma were used to demonstrate an indirect effect of irradiation on the desoxypentose nucleic acid turnover rate. In 2 experiments the animals were irradiated by means of radioyttrium colloids which localize in the liver when injected intravenously and remain at the site of injection when given intramuscularly. The nucleic acid turnover rate was measured by giving a tracer dose of radioactive sodium phosphate, sacrificing the ani-

mals after 2 hours, and isolating the desoxypentose nucleic acid from their tumors and livers. The specific activity of the nucleic acid phosphorus was measured and considered to be an index of the turnover rate. It was found necessary to purify the nucleic acid much more carefully than is done in standard methods in order to obtain a constant specific activity upon successive reprecipitations.

The animals whose livers were irradiated with 4.25×10^5 ergs showed only 66% of the tumor nucleic acid specific activity of the control group. The animals whose muscles were irradiated with 5.25×10^5 ergs showed 84% of the tumor nucleic acid specific activity of the control group. Both these experiments definitely confirm the existence of an indirect effect of radiation on the desoxypentose nucleic acid turnover rate.

One group of animals received 60 r (1.4×10^5 ergs) total body X-irradiation with 180 KV X-rays and both the tumors and livers showed a significant depression in the turnover rate.

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Further Experiments on Influence of Hyaluronidase on Formation of Intraperitoneal Adhesions in the Rat. (17951)

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Chandy and Rhoads reported a reduction in the number of adhesions formed in the peritoneum of the rat in response to standardized trauma when a hyaluronidase preparation was employed locally(1). In this study, hyaluronidase was first applied to crush injuries of the rat cecum on the hypothesis that it might diffuse whatever substances that were responsible for initiating the process of adhesion formation due to its action as a spreading factor(2,3,4). Experiments to extend this series to other types of trauma, and other preparations of hyaluronidase have been carried on in this laboratory since with the results that will be recorded below. The additional experiments that were done in the same way showed somewhat the same trend, but the difference was less marked and was not statistically significant when considered apart from the original series. When the method for producing the trauma was changed, these hyaluronidase preparations were ineffective in inhibiting the formation of adhesions. In all of the experiments the white rat was employed.

Methods. The abdomen was opened under aseptic conditions in 110 rats and the cecum traumatized by the use of one of 3 instruments. An Allis tissue forceps was used in 44 animals, a Payr clamp in 19 animals, and a straight Kelly hemostat in 47 animals. After the injury had been produced, an observer decided whether the animal was to be injected locally with hyaluronidase or used as a control. Schering hyaluronidase was applied to the Allis and Payr clamp injuries and Hydase (Wyeth) and Alidase (Searle) to straight hemostat injuries (see Table).

Discussion. An analysis of the results obtained reveals that adhesions developed in 76% of 55 animals treated with various preparations of hyaluronidase after standardized mechanical methods of producing adhesions were performed. These methods at the same time produced adhesions in 89% of the 55 animals that were used as controls.

1. Chandy, J., and Rhoads, J. E., *Fed. Proc.*, 1946, v5, 218.

2. Chain, E., and Duthrie, E. S., *British J. Exp. Path.*, 1940, v21, 324.

3. Meyer, K., Chaffee, E., Hobby, G. L., Dawson, M. H., *J. Exp. Med.*, 1941, v73, 309.

4. McClean, D., and Hale, C. W., *Biochem. J.*, 1941, v35, 159.

TABLE I.
More Recent Experiments on Effect of Hyaluronidase on Adhesion Formation Due to Mechanical Trauma.

Agent (applied locally)	Total	% adhesions in treated rats	% adhesions in control	Instrument used to produce adhesions
5% hyaluronidase (Schering)	19	42.1	76	Allis
5% hyaluronidase (Schering)	9	88.9	100	Payr
1250 TRU hydase (Wyeth)	17	94	100	Kelly
1250 VRU alidase (Searle)	10	100	100	"

TRU = Turbidity reducing units.
VRU = Viscosity reducing units.

While it is not possible with the data now available to prove that hyaluronidase will not exert an effect under some other experimental conditions, our inability to extend the original observations in this series of experiments has led us to believe that hyaluronidase, in the rather liberal doses used, has no general applicability in the prevention of intraperitoneal adhesions in the rat.

Conclusions. Adhesions were produced in the rat by mechanical trauma to the wall of the cecum by 3 standardized methods. The effect of 3 hyaluronidase preparations applied locally to the injured area at the time of laparotomy was tested. One preparation was tested against 2 types of injury and the other 2 preparations were tested against a third type of injury. No statistically significant effects were obtained to support the positive results previously reported by 2 of us.

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Polysaccharides in Ulcerative Colitis and Normal Human Rectum Compared by Stains of Biopsy Specimens. (17952)

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The hypothesis of Meyer, Gellhorn, Prudden, Lehman and Steinberg(1) that the enzyme lysozyme(2) plays a role in the pathogenesis of chronic ulcerative colitis depends in part on the demonstration of a lysozyme substrate in the colon. These workers found extremely high titers of lysozyme in the feces of patients with chronic ulcerative colitis. We have also observed an elevated output of lysozyme in the feces of all patients with ulcerative colitis tested so far. As yet, Meyer (1), Jerzy Glass(3), and others(4) have been unable to demonstrate a lysozyme substrate in the bowel or in the surface mucus from the colon. Wang and coworkers(4) report damage of the mucosa and submucosa by lysozyme placed in the lumen of the bowel of rats. This report concerns the demonstration of a lysozyme substrate by the histochemical method of McManus(5) or Hotch-

1. Meyer, K., Gellhorn, A., Prudden, J. F., Lehman, W. L., and Steinberg, A., (a) PROC. SOC. EXP. BIOL. AND MED., 1947, v65, 221; (b) *Am. J. Med.*, 1948, v5, 496.

2. Fleming, A., and Allison, V. D., *Brit. J. Exp. Path.*, 1922, v3, 252.

3. Jerzy Glass, G. B., Pugh, B. L., Grace, W. J., and Wolf, S., *J. Clin. Invest.*, 1950, v29, 12.

4. Wang, K. J., Grant, R., Janowitz, H. D., and Grossman, M. I., *Arch. Path.*, 1950, v49, 298.

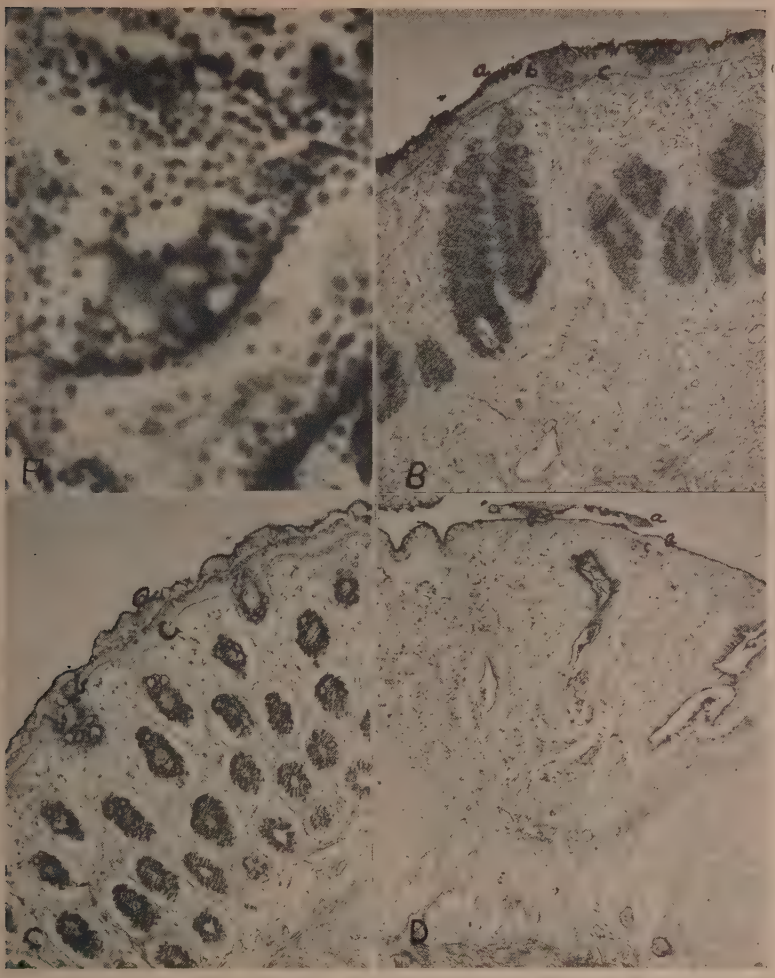

FIG. 1A, B, C, D. (All McManus stain only).
(A) *Micrococcus lysodeikticus* fixed in gelfoam, infiltrated with paraffin, sectioned and stained by the McManus method. (1367×). (B) Normal rectum stained by the McManus method. (80×) (a—surface polysaccharides; b—intracellular accumulations; c—basement membrane). (C) Section of rectum from a patient with chronic ulcerative colitis. (80×) (a—surface polysaccharides; b—intracellular accumulations; c—basement membrane). (D) Section of rectum from a patient with chronic ulcerative colitis. (80×) (a—surface polysaccharides; b—intracellular accumulations; c—basement membrane).

kiss(6) and investigations to determine whether or not this substrate is present in human rectum.

Method. Surface growth of the organism, *Micrococcus lysodeikticus,* was embedded in gelfoam and fixed in 10% formalin, 100% alcohol and acetone. The 3 specimens were infiltrated with paraffin and 6-12 μ sections were cut. Sets of sections from each fixative were attached to slides with gelatin and egg

5. McManus, J. F. A., *Nature,* 1946, v158, 202.
6. Hotchkiss, R. D., *Arch. Biochem.,* 1948, v16, 131.

albumin respectively. The slides were kept at 60°C for 1 hour, the paraffin removed and stains applied. Biopsies from areas of bowel with active disease were taken at proctoscopy in 10 patients with ulcerative colitis. Biopsies were similarly taken from the bowel of patients without organic disease. Some of these specimens were put in lysozyme (0.5 mg crystalline egg white lysozyme / cc saline for 12 hours) before and after fixation. All specimens were fixed in 10% aqueous formalin, 100% alcohol or acetone. After fixation, sections were made and stained with the McManus or Hotchkiss technic. Hematoxylin and eosin preparations also were made. Fixed tissues were also incubated in hyaluronidase (50 TRU/cc buffer pH 6.5) for 20 hours. Amylase was used to prevent the staining of glycogen.

Results. (1) Surface growth of *Micrococcus lysodeikticus* as prepared by this method revealed the organisms to be brilliantly stained with the McManus or Hotchkiss method for polysaccharides. This was true for all methods of fixation and whether the sections were attached to the slide with gelatin or with egg albumin (Fig. 1A).

(2) Treatment of deparaffinated sections of these prepared organisms with lysozyme revealed a complete loss of the ability of the organisms to take on this polysaccharide stain, whereas the saline control sections showed no loss of the polysaccharides. This was found to be true for specimens prepared in all fixatives.

(3) Polysaccharide substances are present in the normal rectum as: (a) a well-defined surface layer, (b) a basement membrane, (c) dense intracellular accumulations in the mucosa, (d) part of the connective tissue fibers, muscle bundles and vascular walls (Fig. 1B).

(4) The surface layer and intracellular accumulations in the mucosa in sections of ulcerative colitis do not appear to differ from the normal (Fig. 1C). The basement membrane in the 10 specimens of ulcerative colitis appeared undisturbed with one exception, in which it seemed much fainter and thinner than normal (Fig. 1D).

(5) Lysozyme applied to normal and ulcerative colitis specimens before fixation and after fixation and sectioning, produces no demonstrable change in the polysaccharides as measured with the McManus-Hotchkiss stain. A biopsy taken from the same individual as was the specimen in Fig. 2 was incubated in lysozyme before fixation and after fixation and sectioning. It appeared identical to the specimen in Fig. 2.

(6) Incubation of bowel sections in hyaluronidase produced no demonstrable changes in stained tissue.

Comments. A specific substrate of lysozyme can be easily demonstrated with the McManus-Hotchkiss stain for polysaccharides. It has been shown with this histochemical method that the substrate disappears after treatment with lysozyme. Many polysaccharide substances are demonstrable in the bowel but none of these are destroyed by lysozyme used in the same manner as that employed to destroy the specific substrate found in the *Micrococcus lysodeikticus*. These observations suggest that the human rectum contains no specific substrate for the enzyme lysozyme.

Summary. 1. The lysozyme substrate contained in *Micrococcus lysodeikticus* is stained by the Hotchkiss or McManus technic after fixation and sectioning by the same procedure utilized for tissue. After fixation and sectioning, the substrate remains susceptible to lysis by lysozyme. Egg albumin, as used in application of sections to slides, does not lyse the lysozyme substrate.

2. Biopsy specimens of rectal mucosa from normal persons and patients with active ulcerative colitis after various methods of fixation show no consistent difference in reaction to the Hotchkiss or McManus stain.

3. *In vitro* application of lysozyme and hyaluronidase to human rectal tissue of normal individuals and patients with ulcerative colitis does not alter the reaction of the tissue to the Hotchkiss or McManus stain.

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Metabolism and Excretion of Estrone Sulfate Labeled with Radioactive Sulfur (S^{35})* (17953)

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Two mechanisms can be postulated to explain the specificity of action of the estrogenic substances on such organs as the vagina, uterus, oviducts, mammae and pituitary. These reactive organs may have the ability of concentrating estrogenic compounds from the circulating blood, or, alternately, the estrogenic susceptible tissues may prove to be unusually sensitive, the characteristic reactions resulting directly from the minute amounts of the circulating hormone without especial concentration of the hormone by the tissue involved.

Because of the many technical difficulties in the extraction and analysis of the minute amounts of the estrogenic steroids which are necessary to produce tissue reactions, little is known concerning the localization and distribution of estrogenic compounds in the organism. With the recent development of tracer technics with radioactive isotopes, a new approach to the problems of estrogen metabolism has been made available. The development of these methods permits the determination of physiologic quantities heretofore impossible by ordinary chemical analyses. Estrone sulfate labeled with S^{35} is not the most desirable compound to employ in a study of some of the aspects of estrogen metabolism inasmuch as the sulfate can readily be removed from the estrone molecule by hydrolysis. However, since estrone sulfate is a naturally occurring substance which possesses estrogenic potency, tracer studies with it might be expected to reveal some of the characteristics of the metabolism of this substance, its localization in tissues, and its susceptibility to hydrolysis by the organism.

Materials and methods. A total of 24 female rats of inbred Sprague Dawley stock maintained in our laboratory were used in this study. Three types of experiments were performed. In the first, 4 rats were injected intravenously with radioactive estrone sulfate. The second group of experiments consisted of 10 animals. In this group the labeled hormone was administered subcutaneously and the animals sacrificed 17 hours later. In both groups selected tissues were analyzed for total radioactivity and extracted for radioactive estrone sulfate. In a third experiment 10 animals received a single subcutaneous injection of varying doses of the radioactive hormone. In these animals the urinary and fecal excretion of total and estrone sulfate radioactivity was determined. The easily measured radioactivity of the S^{35} labeled estrone sulfate afforded a convenient method for studying the completeness and specificity of procedures for the extraction of estrone sulfate added to biological material. The procedure described below was found to give quantitative recovery of added radioactivity when 0.2 mg or more of non-radioactive estrone sulfate was added to the sample before extraction. Extraction procedures without added carrier gave less uniform results.

The methods used for analysis are as follows. The tissue and excreta to be analyzed are weighed and a 20% homogenate is made using distilled water. One cubic centimeter of the 20% homogenate is dried on a 10 cm^2 copper plate for counting(1). A 1 cc aliquot of a known dilution of the radioactive estrone sulfate solution used for injection is prepared for counting in a similar manner. Another aliquot of the homogenate is extracted for estrone sulfate in the following manner. Two-tenths (0.2) mg of non-radioactive estrone sulfate is added to the homogenate as a car-

* This work was aided by the Dr. Wallace C. and Clara Abbott Memorial Fund and the Douglas Smith Foundation for Medical Research of the University of Chicago.

1. Kelsey, F. E., *Science*, 1949, v109, 566.

TABLE I.
Average % of Injected Dose Recovered/g Tissue.

Organ	Intravenous after 10 and 15 min.		Subcutaneous after 17 hr	
	Direct count	After extraction	Direct count	After extraction
Blood	.9	.8	.03	.01
Heart	.1	.1	.02	.02
Liver	.4	.1	.04	.02
Kidney	2.3	1.1	.20	.06
Spleen	.1	.1	.05	.04
Uterus vagina	.5	.2	.07	.02
Ovaries	.2	.1	.16	.05
Adrenals	.2	.1		
Urine			21.9	13.3
Feces			4.7	.7
Small intestine			.4	.3

rier. The homogenate is transferred to a 125 cc separatory funnel and 95 cc of absolute methanol is added after rinsing the homogenizer several times with 25 cc portions of methanol. Twenty-five grams of anhydrous sodium sulfate is added to the mixture and the separatory funnel is shaken for 2 minutes. The material is then filtered with suction through a sintered glass filter. The precipitate is washed with rinsings of the separatory funnel, two 25 cc portions of absolute methanol being used. The filtrate is then concentrated employing gentle heat to approximately 5 cc. The volume is accurately measured and a 1 cc portion is plated for counting. The activity (cpm/mg) of radioactive estrone sulfate in the original sample is determined(1). The total radioactivity of the tissue is determined by counting the tissue homogenate. This represents the tissue content of estrone sulfate plus the radioactive sulfate derived from the hydrolysis of the radioactive estrone sulfate administered to the animal. The radioactivity of the extracted estrone sulfate is a measure of the unhydrolysed material present in the tissue examined.

Results. I. *Intravenous Injection of Radioactive Estrone Sulfate.* Four female rats weighing between 150-200 g were used in this experiment. Eight milligrams of the radioactive estrone sulfate was injected intravenously into the saphenous vein which was exposed under ether anaesthesia. Two animals were sacrificed after an interval of 10 minutes; the remaining 2 after 15 minutes. The following tissues were treated according to the foregoing procedures: blood, heart, liver, kidney, spleen, uterus, and vagina, ovaries and adrenals. In all four animals the greatest concentration of S^{35} was found in the kidneys. Other tissues contained much smaller amounts. As early as 15 minutes after the injection there was some indication of concentration of the radioactivity in the liver and genital tract. There was also some indication that considerable hydrolysis of the estrone sulfate had occurred. With the exception of the blood, heart, and spleen there were marked differences between the amounts of S^{35} present in the whole tissue and that present in the extracted estrone sulfate fraction (Table I).

II. *Subcutaneous Injection of Radioactive Estrone Sulfate.* Ten female rats were injected subcutaneously with a dose of 8.5 mg of radioactive estrone sulfate. The animals were sacrificed for analysis 17 hours after injection. Urine and feces were collected and analyzed.

The tissues analyzed showed no great tendency to localize the radioactivity. As in the intravenous experiment the kidney showed the highest concentration of radioactivity in both total and extracted fractions. Also as in the preceding experiment there is evidence of hydrolysis of the estrone sulfate.

The urine contained 21.9% of the injected dose of radioactivity in this period while only 4.7% was found in the feces. As with the tissues the recoveries from urine and feces indicate marked hydrolysis of the hor-

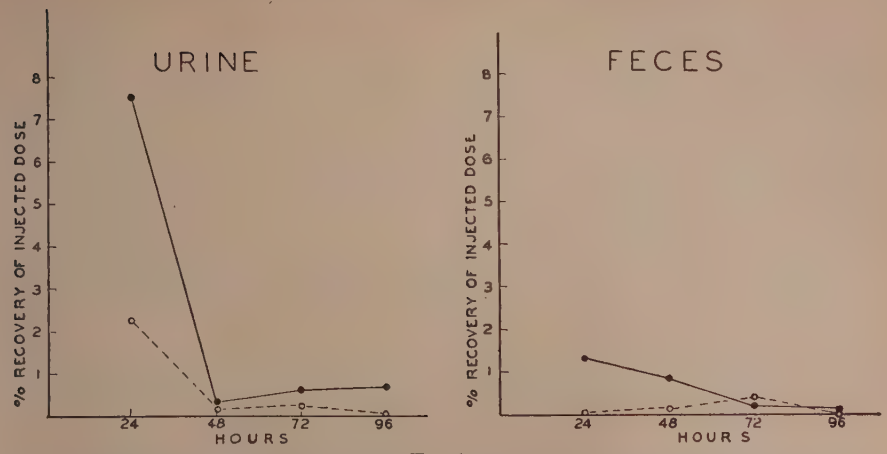

FIG. 1.

Average rate of excretion in 2 animals each receiving 0.2 mg of the labeled hormone. Note that the greatest amount of radioactivity after extraction was excreted in the urine during the first 24 hr. During this interval no radioactivity after extraction was found in the feces. ●——● total radioactivity; ○ - - - ○ radioactivity after extraction.

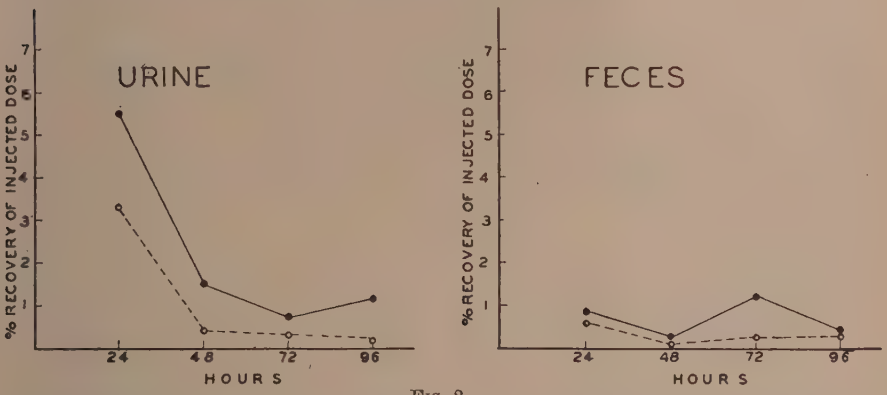

FIG. 2.

Average rate of excretion in 3 animals each receiving 10 mg of the labeled hormone. In this case also the greatest amount of radioactivity after extraction was excreted in the urine during the first 24 hr. ●——● total radioactivity; ○ - - - ○ radioactivity after extraction.

mone (Table I). The animals uniformly showed marked distension of the uterus as a result of the injection.

III. *Excretion of Estrone Sulfate.* Ten female rats weighing approximately 150 g were injected subcutaneously with a single dose of the hormone. The rate of excretion at 24 hour periods following various dosages was determined. Following the excretion study the animals were sacrificed, the uterus and small intestine were analyzed for retained radioactivity. None was found.

Two animals receiving 0.2 mg of the hormone completely eliminated the measurable radioactivity at the end of 96 hours. During this period 27.5% of the total radioactivity was recovered. When the excreta was extracted only 9% of the radioactivity was found to be in combined form. The highest percent recovery was during the first 24 hour period. During this period the feces contained no extractable radioactive estrone sulfate (Fig. 1).

Three animals received a single 5 mg dose

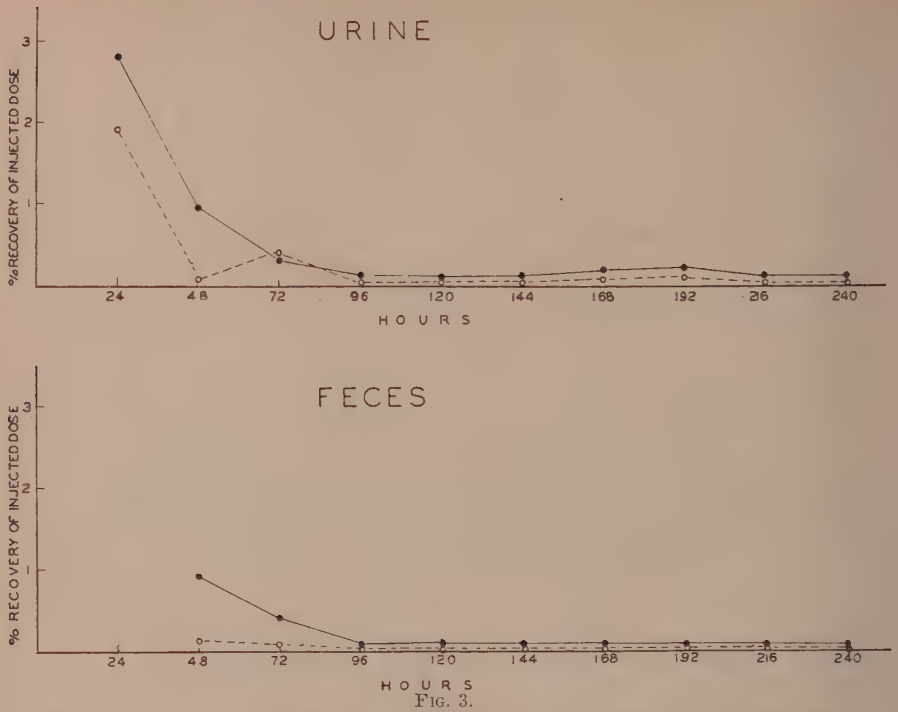

FIG. 3.

Excretion rate in a single animal receiving 250 mg of the labeled hormone. Note that the greatest percentage of radioactivity was excreted in the first 96 hr. This was followed by a relatively constant rate of excretion up to 288 hr. ●——● total radioactivity; ○---○ radioactivity after extraction.

of estrone sulfate subcutaneously. Measurable excretion was completed within 144 hours. In these animals 41% of the total radioactivity was recovered while 19% of the injected dose was extractable as estrone sulfate.

Three additional animals received a single dose of 10 mg of estrone sulfate. In these animals the total radioactivity recovered was 73%. The extractable portion was 47% (Fig. 2).

After the measurable excretion was completed attempts to find additional radioactivity in the uterus and small intestine failed.

A single animal received 250 mg of the hormone subcutaneously in one dose. Excretion of radioactivity continued for 288 hours. Fifty-three percent of the total dose was recovered but only 10% in the combined form (Fig. 3). Another animal receiving a single subcutaneous injection of 500 mg developed convulsions and died after 192 hours. All animals receiving the hormone showed characteristic signs of estrus.

Discussion. The results presented indicate that there is no marked ability of the estrogen sensitive organs to concentrate estrone sulfate. This finding is in harmony with that of Frank(2) who demonstrated that normal female muscle contained as much estrogenic activity as fibromyomatas of the uterus. More recently Twombly *et al.*(3) using radioactive dibromestrone were unable to demonstrate localization of this substance in any target organ. The same findings are reported by Albert, *et al.*(4) who used iodin-

2. Frank, R. T., PROC. SOC. EXP. BIOL. AND MED., 1935, v32, 1665.

3. Twombly, G. H., McClintock, L., Engelman, M., *Am. J. Obst. and Gynec.*, 1948, v56, 260.

4. Albert, S., Heard, R. D. H., Leblond, C. P., and Saffran, J., *J. Biol. Chem.*, 1949, v177, 247.

ated derivatives of estradiol. These latter two reports dealt with substances which had little or no estrogenic activity. The estrone sulfate used in these experiments had marked estrogenic potency but still did not show any marked localization in the estrogenic susceptable tissues, the highest concentration encountered was in the kidney which is one of the routes of excretion. This is borne out by the high amount found in the urine. That some of this hormone is excreted by way of the gastrointestinal tract is demonstrated by the presence of radioactive estrone sulfate in the small intestine and feces. This observation substantiates the finding of Levin(5) who reported large amounts of estrogen in the feces of pregnant cows.

Summary and conclusions. 1. Radioactive estrone sulfate shows no definite localization in any of the target organs following intravenous or subcutaneous injection into mature female rats in spite of marked estrogenic response. 2. Estrone sulfate is rapidly hydrolyzed in the body. 3. Estrone sulfate is excreted not only by way of the urinary tract but considerable amounts find their way into feces.

We wish to thank Dr. Edward C. Reifenstein, Jr., and Ayerst, McKenna and Harrison for the labeled and non-labeled estrone sulfate which they generously supplied.

5. Levin, L., *J. Biol. Chem.*, 1945, v157, 407.

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Antibiotic Studies on Beta Hemolytic Streptococci: VII. Acquired *in vitro* Resistance to Bacitracin* (17954)

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Acquisition of bacitracin resistance by beta hemolytic streptococci has not been reported to date. Meleney(1) found no increase in resistance to bacitracin in 5 strains of group A beta hemolytic streptococci after 26 serial transfers on bacitracin agar. Stone(2) studied 4 strains of *Staphylococcus aureus* and reported an increase in resistance of a maximum of 125-fold in one strain after 62 exposures to bacitracin and a minimum of 3-fold in another strain after 35 exposures. The resistance decreased rapidly on serial transfers in bacitracin-free broth but was stable on storage in a refrigerator for a period up to 29 days.

The present study was undertaken (1) to determine whether or not streptococci of groups A, B and C would develop bacitracin resistance in the manner similar to that observed for other antibiotics and (2) to determine whether or not bacitracin altered the biology of streptococci in a manner similar to that produced by penicillin(3-6), streptomycin(7), and aureomycin(8).

Materials and methods. The 23 strains of beta hemolytic streptococci, 8 of group A, 8

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Bacitracin was supplied by the Commercial Solvents Corporation, through the Antibiotics Study Section of the National Institute of Health.

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TABLE I.
Bacitracin Sensitivity of Group A, B, and C Beta Hemolytic Streptococci Isolated in the Period from December 7, 1948 to March 22, 1949.

Units per ml	Group A strains		Group B strains		Group C strains	
	No.	%	No.	%	No.	%
.003	116	57				
.005	66	32	4	18	27	54
.01	22	11	6	27	17	34
.1			7	32	5	10
.5			3	14	1	2
1			2	9		
Total	204		22		50	

of group C, and 7 of group B, previously described(3-8) were again employed. Bacitracin sensitivity was determined by the Fleming(9) ditch plate method. All of the bacitracin used for the induction of resistance was from a single lot. Some of the subsequent studies were performed with bacitracin of two other lots that had been standardized against the original material. The antibiotic was made up at 3 to 5 day intervals in physiological saline at a pH of 6.8 and stock solutions stored in the refrigerator. To induce resistance *in vitro*, 40 subcultivations were made on blood agar containing graded concentrations of bacitracin by the method previously described(3,8). Comparison was made of the mouse virulence of the parent strain, the control strain (parent strain after 40 serial transfers on blood agar plates containing no antibiotic), the induced resistant strain and the resistant variant after 12 to 14 passages in normal mice by an intracerebral inoculation method(3,8). Serial dilutions were prepared in broth and pour plate colony counts determined on each strain. The methods used for the determination of streptolysin "S", streptokinase, proteinase, and ribonuclease activity have been described previously(10). The desoxyribonuclease activity of the streptococci was measured by the turbidimetric method described by McCarty(11).

Results. A. *Initial bacitracin sensitivity.* Table I shows the bacitracin sensitivity of 276 recently isolated beta hemolytic streptococci of groups A, B, and C. The sensitivity varied from 0.003 to 1 unit per ml. The range of sensitivity within groups increased in the order B greater than C greater than A.

B. *Acquired resistance on bacitracin media.* The minimum increase in resistance was 8-fold in a group A organism; the maximum increase was 3,750-fold in a group B organism. In general there were wide differences in the rate of development of resistance within all 3 groups but the extreme variations were most evident in group B. This is clearly demonstrated by comparing the mean and median fold increase in resistance by groups. There appears to be no characteristic pattern in the group behavior. The results are summarized in Table II.

C. *Acquired resistance on control media.* The 23 parent strains were subcultured serially 40 times on bacitracin-free blood agar. No change in bacitracin resistance was evident at the end of these serial subcultivations and no colonial or hemolytic changes were noted.

D. *Loss of acquired resistance.* Three bacitracin-resistant strains of group A, 3 of group B, and 3 of group C were transferred serially 100 times on bacitracin-free blood agar plates and simultaneously 12 to 14 times intracerebrally in normal mice. The organisms were retested by the whole plate method on the 50th and 100th serial plate transfers and at the end of 12 to 14 serial mouse passages. In addition, these strains were tested after 20 months storage in blood infusion broth at 4°C. During this period of 20 months, 2 subcultivations had been made into fresh blood

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TABLE II.
Bacitracin Resistance Induced in Group A, B, and C Beta Hemolytic Streptococci After 40 Subcultivations on Bacitracin Medium.

Group	No. strains	Range fold incr.	Mean fold incr.	Median fold incr.
A	8	8- 100	43	40*
B	7	15-3,750	596	50
C	8	12- 300	99	50*

* Approx. values.

TABLE III.
In vitro Sensitivities of Bacitracin-Resistant Beta Hemolytic Streptococci After Serial Transfers on Control Medium, Storage in Broth at 4°C or Passage Through Mice.

		Mean sensitivity u/ml				
Group	No. strains	Parent	Resistant	Plate transferred 100 χ s	Stored at 4°C	Mouse-passed, 12 χ s
A	5	.01	.5	.01	.02	.01
B	5	.7	32	5	4	2
C	3	.03	7	2	1	1

TABLE IV.
Mouse Virulence of Beta Hemolytic Streptococci with Acquired Bacitracin Resistance.

			LD_{50}* after 40 transfers		LD_{50}* of resistant strain after 12 mouse passages
Group	Strain	Initial LD_{50}*	Control medium	Bacitracin medium	
A	S2	1.6×10^2	1.3×10^4	2.7×10^3	1.8
	4	3.7×10^2	3.8×10^3	3.4×10^4	1.5×10^4†
B	T5	1.9×10^4	1.2×10^2	$>1.2 \times 10^7$	2.2×10^5
	8	7.8×10^2	7	4.9×10^4	1.6×10^3
C	U1	2.1×10^5	4.9×10^4	5.1×10^4	2.2×10^4†
	2	9.3×10	2.5×10^3	1.1×10^6	2.8×10^5

* The LD_{50} is expressed in terms of the number of viable streptococci injected intracerebrally.
† Virulence determined after 14 mouse passages.

broth. These results are recorded in Table III. It is evident that acquired bacitracin resistance is a fairly labile characteristic of streptococci and that cold storage as well as normal plate or mouse passage permits a decrease in resistance. Three of 3 resistant group A strains returned to the level of the parent sensitivity by the 100th serial transfer on control media. Two of 5 lost all while the remaining 3 lost part of the acquired resistance on storage for a period of 20 months. Three of 3 lost all acquired resistance on serial passages through normal animals. The group B and C resistant organisms were easily resensitized by any of the 3 methods but this was to a lesser degree than those of group A.

E. *Change in virulence.* The mouse virulence of 9 organisms was studied before and after induced resistance. The results in representative strains are given in Table IV. Decreased virulence accompanied acquired resistance in 7 of 9 strains. The change varied from approximately 10-fold in 2 organisms to approximately 10,000-fold in 1 strain. Twelve to 14 serial passages in normal mice partially or completely restored the virulence in 6 of 9 strains.

F. *Antigenic and hemolytic changes.* The 23 beta hemolytic streptococci were regrouped by the Lancefield technic after acquiring bacitracin resistance. All group A and C strains remained group specific. Three of the 7 group B strains could not be grouped after induced resistance. The parents of the S_6 and U_6 strains were the only group A strains which could be typed by the precipitin technic.†

† These results were confirmed by Naval Medical Research Unit No. 4.

TABLE V.
Streptolysin "S" Production by Bacitracin-Resistant Beta Hemolytic Streptococci.

		Parent		Control‡		Bacitracin-resistant		Resensitized by 50 or 100 plate transfers		Resensitized by 16 mouse passages	
Group	Strain	Units*	Density†	Units	Density	Units	Density	Units	Density	Units	Density
A	S2	100	69	100	66	20	79	100	67	100	67
	3	100	66	100	66	20	75	100	67	65	67
	4	65	69	50	71	65	68	—	—	65	59

* Hemolytic units are expressed as the reciprocal of dilution of lysin where 50% hemolysis of red blood cells was observed after 30 min. incubation at 37°C.

† Density of growth is expressed as % light transmission at λ 600 γ on a Coleman, Jr., spectrophotometer.

‡ Control organism are parent strains after 60 transfers on plain blood agar.

The S_6 strain was a type 43 and the U_6 a type 18 organism. At the end of 40 transfers on bacitracin medium the resistant variant of the S_6 strain was no longer typable while the U_6 resistant organism remained type-specific. On repeating this the S_6 strain has remained typable through 31 transfers of the parent organism on bacitracin medium. This work is continuing. Transient conversion from beta to alpha hemolysis occurred on sub-inhibitory concentrations of bacitracin in most of the 23 strains. Minute colonies also appeared when inhibitory concentrations of bacitracin were approached.

G. *Enzymatic*‡ *changes.* The enzyme changes observed in streptococci after acquired bacitracin resistance have been published(10). The same strains, S_2, S_3 and S_4, after storage for approximately 20 months at 4°C, were transferred an additional 20 times on bacitracin medium to restore the lost resistance. These organisms were then assayed for (a) proteinase-streptokinase activity, (b) streptolysin "S" titre and (c) ribo- and desoxyribonuclease activity. No differences were observed when comparisons were made of enzymatic activity of resistant strains grown in the test broth with and without sub-inhibitory concentrations of bacitracin. The streptolysin "S" titre was lowered in 2 of 3 bacitracin-resistant streptococci assayed after 60 antibiotic transfers (Table V). However, these two strains also showed decreased density of growth measured spectrophotometrically, as compared with their controls. The relationship between titre and density of growth has not been evaluated. Equal or higher enzyme activity has been produced by variants resistant to other antibiotics when their growth was less than that in corresponding controls(12). At the 60th transfer, the production of ribo- and desoxyribonucleases by the S_2, S_3 and S_4 bacitracin-resistant variants as well as their proteinase-streptokinase ratios remained unchanged.

Discussion. Resistance to bacitracin can be induced in beta hemolytic streptococci with relative ease as compared with penicillin or aureomycin. The technics are comparable. Large single-step increases in resistance such as were seen with streptomycin do not occur. The induced bacitracin resistance of streptococci is the most labile antibiotic resistance we have studied. The alterations in the biology of the streptococcus parallel those with the other antibiotics. Mouse virulence is decreased consistently and the changes from beta to alpha hemolysis were seen in almost all of the strains studied. The changes in the enzyme systems for the most part were minimal.

Summary. 1. The bacitracin sensitivity range of 276 strains of groups A, B and C hemolytic streptococci was 0.003 to 1 unit/ml. 2. Bacitracin resistance was induced in 23 strains of groups A, B, and C streptococci by 40 serial transfers on bacitracin-agar. The increase in resistance ranged from 8-fold in a group A to 3,750-fold in a group B organism. The median fold increase in resistance was comparable for all 3 groups. 3. Serial trans-

‡ Streptolysin "S" is included for simplicity of discussion although this is an unproved enzyme system.

12. Unpublished data.

fers on control media, passages through normal mice and storage at 4°C partially or completely restored the bacitracin sensitivity to all resistant variants. 4. Mouse virulence was decreased in 7 of 9 strains. It was restored in 6 of the 7 strains by subsequent passages in normal mice. 5. Group specificity was lost in 3 of 7 group B strains after induced resistance. 6. Growth on sub-inhibitory concentrations of bacitracin stimulated transient conversion from beta to alpha hemolysis in most strains studied. 7. The streptolysin "S" titre was decreased in 2 of 3 resistant variants. Ribo- and desoxyribonuclease activity and the proteinase-streptokinase ratios remained unchanged after acquired resistance.

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The Cultivation of Coxsackie Virus.*† (17955)

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Recent laboratory studies(1-6) have established a newly discovered(1) group of viruses, the Coxsackie or "C" group, as the etiological agents responsible for human disease diagnosed clinically as nonparalytic poliomyelitis (1-4,7), "summer grippe"(5), aseptic meningitis(3) and epidemic myalgia(4). The viruses have been recovered from feces and throat swabs of patients and from sewage and flies(3). This group of viruses is characterized by its pathogenicity for newborn mice and hamsters with resultant myositis, paralysis and death.

In the experiments reported herein evidence is presented for the successful propagation *in vitro* in the presence of living tissue cells of a member of the Coxsackie group of viruses, type 4, Minnesota 1 strain.§

Materials and Methods. Virus. The strain of virus was isolated from a fecal specimen which had been obtained on about the 10th day following the onset of the illness of a patient whose diagnosis, both on admission and discharge from the University of Minnesota Hospitals, was nonparalytic poliomyelitis. The feces were made up in 10% suspension, homogenized for 5 minutes in a Waring blender and centrifuged at 10,000 r.p.m. for 20 minutes. The supernatant fluid was removed, filtered through a Seitz-EK sterilizing filter pad and the filtrate was employed for injection intraperitoneally, 0.05 ml, into each member of a litter of Rockland Swiss albino mice within 24-36 hours of their birth. When these infant mice were ill or dead, they were frozen, triturated in a mortar in 0.85% saline to yield a 10% suspension, filtered as described above, and the filtrate, 0.03 ml, was used as the inoculum for passage to infant mice. Following 10 mouse passages in series, 0.03 ml of filtrate similarly prepared was used to initiate each tissue culture passage series.

Tissue Cells. Infant mice killed within about 48 hours before or after birth provided the tissues which were employed to

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† Bact. Proc., Baltimore, May 16, 1950.

‡ Part of the material in this paper will appear in a thesis to be submitted by Mr. Slater in partial fulfillment of the requirements for the degree of Master of Science.

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§ The authors are indebted to Dr. G. Dalldorf for allocating Minnesota strain 1, Coxsackie group, as type 4, subgroup A, Coxsackie group of viruses.

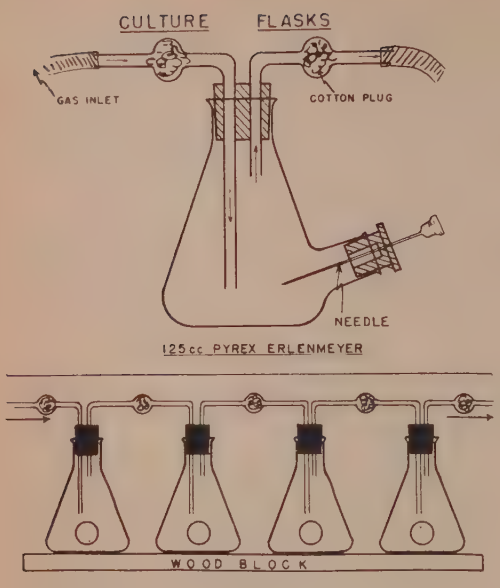

FIG. 1.
Tissue culture flask and flask assembly.

prepare 3 tissue pools made up of cells from (a) intestines, (b) brains, and (c) muscle (a mixture of hepatic, splenic and muscle tissues were used for the first 5 passages). Each tissue-pool was finely minced in Simm's UF/3X7 fluid with sharp iris scissors to a particle size that entered a 10 ml serological pipette. One drop of this tissue pulp and 0.03 ml of virus suspension were added to 5 ml of Simm's UF/3X7 contained in a newly designed tissue culture 125 ml flask (Fig. 1). The utilization of this flask and of Mohr clamps for closure made it possible to pass daily a gaseous mixture of 5% carbon dioxide, 21% oxygen, and 74% nitrogen over the surface of the content, and to maintain an identical atmosphere within each battery of flasks. After incubation had been carried out at 35.5°C for 72 hours, the supernatant fluid was withdrawn from each flask, pooled, and 0.03 ml was employed as the inoculum for each flask in the successive passage serial transfer.

Results. Three passage series, A, intestinal tissue cells; B, brain tissue cells; C, a mixture of muscle, hepatic and splenic cells for 5 passages and, thereafter, muscle alone, had been carried through 24 passage transfers when this report was written. The virus content of the supernatant fluids, as successive tenfold decrements, was determined for each dilution at intervals by the injection intraperitoneally, 0.03 ml, into each of a litter of mice from 1 to 5 days old. The results for 24 passages are summarized in Fig. 2.

It can be seen that the virus through the 12th passage was almost uniformly lethal. The titer after 10 passages was 10^6. When virus representative of the 16th passage was tested, virus was present in a concentration of 10^6 as evidenced by paralysis of the test mice. However, it became apparent that a change in relative pathogenicity had occurred, since many mice survived at all dilutions to give an over-all mortality of 42%. The surviving mice showed marked tremors, spasticity, paralysis and residual stunting. Since the dilution factor after the 24th passage was more than $(166)^{24}$, unequivocal evidence for multiplication of the virus had been effected.

Summary. A Coxsackie virus, Minnesota 1 strain, following its isolation from a fecal sample of a patient with a clinical diagnosis of nonparalytic poliomyelitis, was maintained successfully for 24 successive passages in a tissue medium made up of minced embryonic mouse cells and Simm's UF/3X7 fluid.

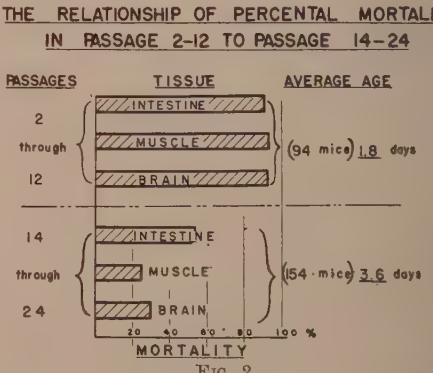

FIG. 2.
The relationship of percental mortality of the newborn mice employed for testing virus after from 2 to 12 passages in tissue culture and of the mice similarly employed for testing virus after from 14 to 24 passages.

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Chronic Toxicity of Thiomerin Compared to Other Mercurial Diuretics.* (17956)

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Mercurial diuretics when administered intravenously offer the definite clinical hazard of producing acute cardiac effects. Attempts to find compounds to minimize or eliminate such reactions led to the clinical introduction of N-(gamma-carboxymethyl-mercapto-mercuri-beta methoxy)propyl camphoramic acid (Thiomerin)(1-6). The initial study of its toxicity was based on acute cardiac effects in which a ratio of 1:160 was expressed in comparison to Mercuhydrin(7). Some of the recent clinical reports concerning Thiomerin indicate that this ratio may be considered as the chief criterion of toxicity(1-3), although other workers acknowledge that it is but one indication of Thiomerin toxicity, *viz.*, its acute cardiac effect(4-6). Other criteria of toxicity obviously should be determined and considered for mercurial diuretics, compounds which are characterized by their rather prolonged action on the kidney. In this respect an increased incidence of systemic reactions to Thiomerin has been mentioned in one clinical report(1). Further, in mice, the intravenous lethal dose of this compound over a 4 day period was found to be of the same order as other diuretics(8). Handley *et al.*(9) demonstrated greater toxicity in the dog with a test of actual renal function, glomerular filtration as determined by creatinine clearances, and by the fact that Thiomerin caused a greater number of deaths. Inasmuch as Thiomerin when administered parenterally causes less local reaction than certain other diuretics(10), the question arose as to whether or not the simple addition of a monothiol compound, *e.g.*, Sodium Thioglycollate, to Mercurophylline or Mercuhydrin would decrease the local toxic reaction of these mercurials. Such a mixture would be similar in composition to Thiomerin since the latter is essentially Mercurophylline Injection U.S.P., in which the theophylline has been replaced by sodium mercapto-acetate(4). In referring to the work of Long and Farah(11), Lehman suggested that mixing a thiol with a mercurial previous to injection might reduce the toxicity of the latter.

The present study was initiated when it was found that mixing Mercurophylline with Sodium Thioglycollate caused a marked increase in the LD_{50} of the mercurial after subcutaneous administration. This report deals with the toxicity of Thiomerin in comparison with other mercurial diuretics and the mixture of Sodium Thioglycollate with Mercurophylline, after subcutaneous or intravenous administration in rats, and with the distribution and excretion of the mercury in the animal.

Methods. Sprague-Dawley albino rats were used as the experimental animal. The drugs

* The animals and supplies used in this study were made available by the Kremers-Urban Co. of Milwaukee.

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TABLE I.
Toxicity in Rats of Mercurial Diuretics Following a *Single* Subcutaneous Administration.

Dosage, total mercury, mg	Mercuhydrin	Mercurophylline			Thiomerin
		Plain	With benzyl alcohol	+ Na thioglycollate	
8.	5/5	10/10	0/10	5/5	12/12
4.	14/20	0/20	0/20	20/20	20/20
2.	0/10			5/20	10/10
1.5					5/15
1.					0/10

The ratio is expressed as: No. of rats killed/No. of rats injected.

tested were Meralluride (Mercuhydrin), Mercurophylline,† Mercurophylline with 2% benzyl alcohol,† Thiomerin, and the combination of equal parts of Mercurophylline† and a 6% aqueous solution of Sodium Thioglycollate. All of the drugs were used on the basis of a mercury content of 40 mg/cc. Inasmuch as Thiomerin is recommended for subcutaneous administration, the other compounds also were given by this route. Similarly, Thiomerin was tested after intravenous injection in order to compare its toxicity with that of mercurials commonly administered in this manner. The compounds were given as *single injections* in the range of doses indicated in Tables I and II. Subcutaneous injections were made into the flank and, with the exception of the doses of Thiomerin containing 1.5 mg and 1.0 mg of mercury, were not diluted before injection. For the intravenous injections the drugs were diluted to a total volume of 2 cc. Acute deaths were considered to be those that occurred during or within the first five minutes after completion of the injection. Delayed deaths were those that occurred after this initial period but in 7 days or less.

Thiomerin and Mercurophylline were used for studies of excretion and distribution of mercury in rats after subcutaneous administration. Dilutions of the 2 drugs were prepared so that 0.5 cc should contain 1.5 mg of mercury. This amount was injected and the rats placed in metabolism cages arranged for collection of urine and feces, as described by Harned, Cunningham and Gill (12). No food was available to the animals but water was permitted *ad lib.* Urine was collected during the survival of the animals which in no instance exceeded 4 days. The mercury content of the kidneys, the urine, the feces, and the carcass of the rat was determined by the dithizone method described by Kozelka(13).

Results. The results of single subcutaneous injections are presented in Table I. While no attempt was made to adjust the subcutaneous dosages to an exact mg/kg basis, the animals tested fell into two weight groups (125 to 175 g or 300 to 350 g) and representative numbers of each group were treated with each drug. It is evident that Mercurophylline with benzyl alcohol was the least toxic of the substances administered subcutaneously since there were no deaths in the group which received the dose of 8 mg of mercury. Thiomerin was the most toxic since the single injection which contained only 2 mg of mercury killed all the animals of that group. The sites of injection were examined for evidence of irritation and it was noted that Thiomerin and the combination of Mercurophylline and Sodium Thioglycollate did not produce local irritation in any dose used. The results following the intravenous administration of a single dose of the compounds are presented in Table II. In this series Mercurophylline was the least toxic, while Thiomerin again was the most toxic. Acute deaths occurred with injections of Mercurophylline and Mercurophylline with benzyl alcohol. In fact, the greatest propor-

† Differs from Mercurophylline Injection U.S.P. in that each cc contains an excess of 10 mg of theophylline.

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TABLE II.
Toxicity in Rats of Mercurial Diuretics Following a *Single* Intravenous Injection.

Dosage, total mercury, mg/kg	Mercuhydrin		Mercurophylline								Thiomerin	
			Plain		With benzyl alcohol		+ Na thioglycollate					
	Acute*	Delayed	Acute	Delayed	Acute	Delayed	Acute	Delayed			Acute	Delayed
36			10	1								
			11									
32			3	1								
			4									
24	0	6	7	5	42	19	0	10			0	10
	6		22		73		10				10	
16			2	1	12	1						
			12		20							
12	0	10			9	1	0	10			0	10
	10				20		10				10	
10	0	9										
	10											
8	0	5					0	8			0	10
	10						10				10	
4	0	0					0	0			0	0
	10						10				10	

The ratio is expressed as: No. of rats killed/No. of rats injected.
* Acute = Deaths within 5 min. after inj.
Delayed = Deaths within 7 days after inj.

tion of deaths at any of the intravenous dosage levels with these two compounds was due to this cardiac effect. The rats died during the injection or within 5 minutes after its completion, usually with convulsions and respiratory distress, even though the drug was given at the rate of 1.0 cc/min. This is in accord with the work of DeGraff and Lehman(14) in which they found that sudden death following the intravenous administration of these drugs cannot be avoided merely by slowing the rate at which they are administered. The production of acute deaths was not experienced with intravenous injections of Mercuhydrin, Thiomerin, or Mercurophylline when combined with Sodium Thioglycollate, and these drugs could be given as rapidly as 1 cc/15 seconds.

Analyses of the rat and its excreta for the distribution of mercury were very inconclusive as to the mechanism of the decidedly increased toxicity of Thiomerin. Actually a quite varied distribution of mercury was found in 9 animals which were analyzed. In general, about 60 to 75% of the administered mercury was excreted in the 2 to 4 days of survival of the animal, although one rat which was given Thiomerin excreted less than 20% of the total in this period. Median retention in the kidneys was only 48 μg of mercury with extremes of 17 and 388 μg. There was no essential difference in distribution between

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the two diuretics used.

Discussion. A higher incidence of delayed deaths occurred from the administration of Mercurophylline to which Sodium Thioglycollate had been added. This substantiated preliminary testing in which it was found that 0.1 cc of Sodium Thioglycollate and 0.1 cc of Mercurophylline (4.0 mg of mercury) when mixed and injected subcutaneously had a greater toxicity than either drug administered alone. Control injections of 30 mg of Sodium Thioglycollate subcutaneously were non-toxic. The toxicity of the combined Mercurophylline and Sodium Thioglycollate following subcutaneous administration is approximately double that of Mercurophylline and is even greater than that of Mercurophylline with benzyl alcohol when given by this route. The toxicity of this combination of mercurial and thiol is, however, of the same order as that of Thiomerin given subcutaneously in rats, a fact which might be anticipated from their similarity of chemical composition. Thus, the addition of the thiol compound, even though it eliminates the danger of acute death following intravenous injection, so increases the chronic toxicity of Mercurophylline by either subcutaneous or intravenous routes that its addition would seem to be a decided hazard.

Summary. Data presented on the comparative toxicity of Thiomerin, Mercuhydrin and Mercurophylline indicate that the compound containing the thiol group definitely is more toxic in rats. Other reports, both clinical and laboratory, have indicated such a possibility. Sodium Thioglycollate potentiates the chronic toxicity of Mercurophylline when combined with it and given by either the subcutaneous or intravenous route. There is, however, elimination of the acute cardiac effect with intravenous administration. Studies of the distribution of mercury in the rat and its excreta following subcutaneous administration of two of the drugs failed to indicate the mechanism of potentiation of toxicity.

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Pancreatic Secretion to Peptic Ulcer. II. Effect of Hypoglycemia With and Without Pancreatectomy.* (17957)

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A definite relationship exists between pancreatic function and peptic ulcer formation. When the alkaline pancreatic secretions are deviated from the duodenum by external pancreatic fistula, ligation of the pancreatic ducts or by implantation of the ducts into the lower reaches of the gut without ablation of the pancreas, peptic ulcers develop spontaneously (1-4). However, if the duodenum is deprived of the alkaline external secretinos by means of pancreatectomy, ulcers form spontaneously only rarely. It was suggested by Poth, Manhoff, and DeLoach(5) that this difference might be due to the fact that the hyperglycemia due to pancreatectomy suppresses secretion of the gastric acid-pepsin factor sufficiently to protect the gastric and duodenal mucosa from ulceration. The experiments reported in this paper are directed toward the evaluation of this assumption.

Experimental method. Twenty healthy, mongrel dogs were divided into 2 equal groups. Their food consisted of 50 g of raw

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TABLE I.
Sample Protocol (Dog No. 9) Showing Insulin Dosage and the Resulting Blood Sugar Levels.

Date	Blood sugar mg % 8 a.m.	12 n.	4 p.m.	PZI insulin units	Remarks
9/14	55			0	Pancreatectomy (normal blood sugar
15	362			3	level before pancreatectomy varied
16	200			3	between 56 and 69 mg %)
17	132			3	
18	241			0	
19	241			4	
20	44	62	129	4	
21	27	45	239	4	
22	29	32	63	4	
23	20	30	30	4	
24	22			4	
25	23			0	Clinically hypoglycemic
26	244	266	332	4	
27	34	53	123	4	
28	16	54	46	4	
29	38	100	139	4	
30	34	39	66	4	
10/1	17			4	
2					Dead. Autopsy. Several small gastric ulcers

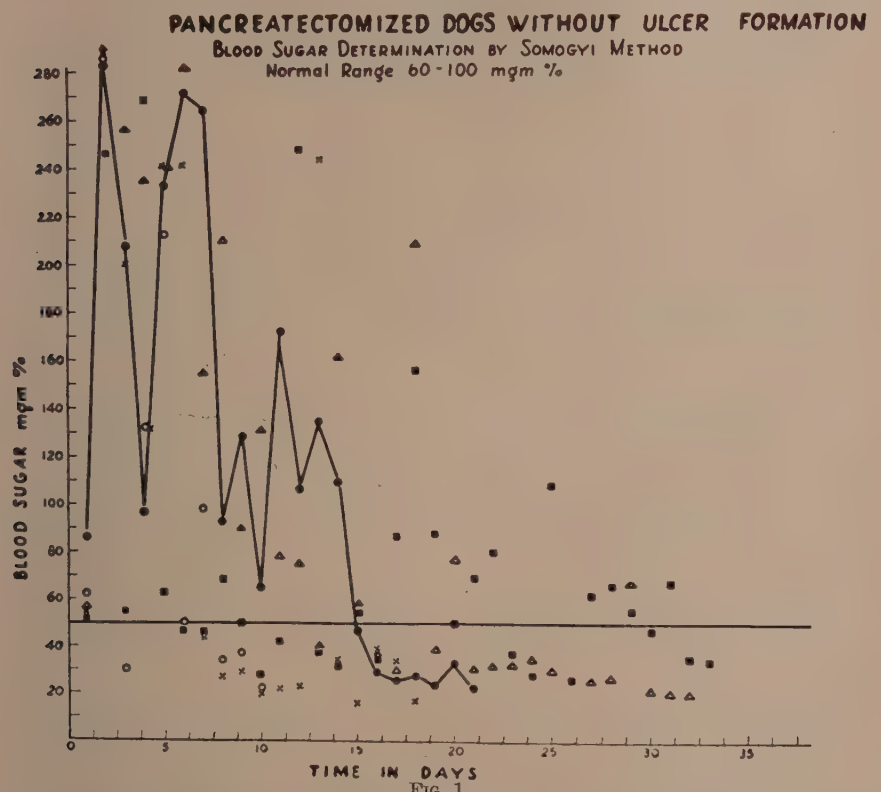

FIG. 1.

Scattergram of blood sugar concentrations of 5 dogs which had the pancreas removed and were then administered commercial protamine zinc insulin in sufficient quantity to reduce the blood sugar level below 50 mg % and eventually result in the animal's death. A curve is drawn through one set of points. There were no peptic ulcers present when these animals died.

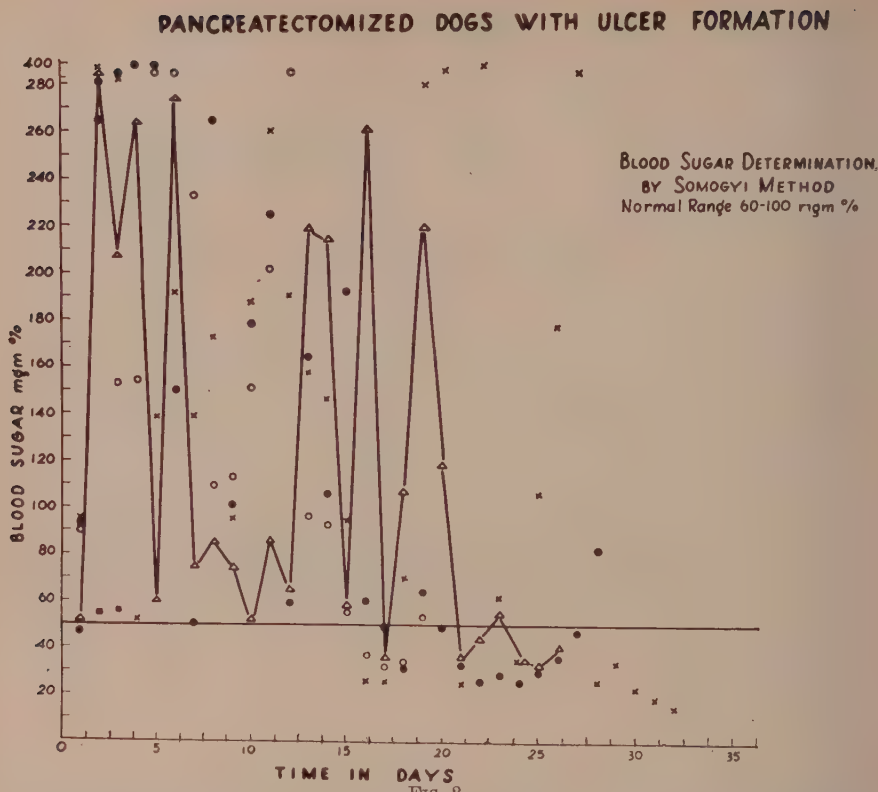

FIG. 2.

Scattergram of blood sugar concentrations of 5 dogs which had the pancreas removed and were then administered commercial protamine zinc insulin in sufficient quantity to reduce the blood sugar level below 50 mg % and eventually result in the animal's death. A curve is drawn through one set of points. Each of these animals developed peptic ulcers.

horse meat daily per kilo of body weight. Initial daily fasting blood sugar levels were obtained during a control period of a few days. One group was then treated with commercial protamine zinc insulin to lower the fasting blood sugar level to 50 mg % or less. The other group was first subjected to pancreatectomy and one gram of choline was added to their daily diets to protect against fatty changes in the liver. Three units of protamine zinc insulin were given daily for five to seven days immediately postoperatively to maintain approximately normal blood sugar levels. Then the dosage of insulin was gradually increased until the fasting blood sugar level was maintained below 50 mg %; preferably in the neighborhood of 40 mg %, Table I. It was difficult to maintain these conditions because an animal might fail to eat all of his food, and also because the animals developed a certain degree of tolerance to insulin. This latter phenomenon was noticed particularly in the animals which had not been pancreatectomized. The administration of insulin was continued until the animals expired. Death frequently resulted from hypoglycemic shock and convulsions. To demonstrate that the changes observed were not due to the convulsions, *per se*, 3 animals were administered metrazol and maintained in a continuous convulsive state for one to three hours. Autopsy performed immediately following the death of these control animals showed the mucosa of the gastrointestinal tract to be normal except for an occasional small, petechial, submucosal hemorrhage. Ulceration had not occurred.

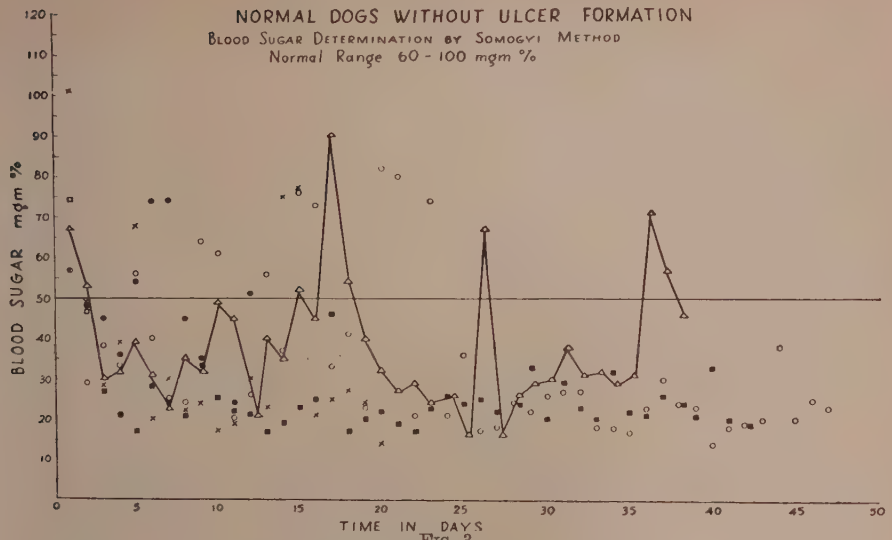

FIG. 3.

Scattergram of blood sugar concentrations of 5 normal dogs which had received commercial protamine zinc insulin in sufficient quantity to reduce the blood sugar level below 50 mg % and eventually result in the animal's death. A curve is drawn through one set of points. There were no peptic ulcers present when these animals died.

Therefore, it is believed that the postmortem ulcers found in the hypoglycemic animals were not due to terminal convulsions.

All animals were examined postmortem for the presence or absence of gastrointestinal ulceration. No residual pancreas could be found in the pancreatectomized animals. The specific data of these experiments are presented in Fig. 1, 2, 3, and 4.

Comparison of the blood sugar levels of the depancreatized animals given in Fig. 1, which failed to form ulcers, with those of Fig. 2, which developed ulcers, shows no appreciable difference in the degree of hypoglycemia. Likewise, the comparison of these data given in Fig. 3 and 4 from similar studies on intact dogs again fails to show any significant difference of blood sugar levels whether or not ulcers developed. Probably all of the animals developed ulcers, but, since they heal so rapidly, they may not be present at time of the animal's death which results from the hypoglycemia.

Discussion. Maintenance of hypoglycemia is rather difficult because of individual variation of susceptibility and because a degree of tolerance to insulin develops. Apparently, the ulceration begins with disruption of the surface cells of the mucosa. This superficial damage is rapidly repaired when the blood sugar level is allowed to return to, or exceed, normal values. Completely reproducible conditions cannot be maintained and might account for the fact that demonstrable ulcers are not produced in all experiments. The incidence of ulcer formation is such, however, as to indicate that hypoglycemia favors the development of peptic ulcers both in the intact animal and in those subjected to pancreatectomy. Peptic ulcers were found at autopsy in half of the animals of both groups.

If hypoglycemia will favor the development of peptic ulcers, then individuals with islet cell tumors of the pancreas showing sustained hypoglycemia should have a high incidence of peptic ulceration. Only a single instance of peptic ulcer accompanying islet cell tumor is found on careful search of the literature. Can one explain this apparent paradox? Commercial insulin is not pure. It may contain as much as 10% of a substance called the H-G factor. This factor causes hyperglycemia and can hydrolyze glycogen. This H-G factor has been isolated from the mucosa

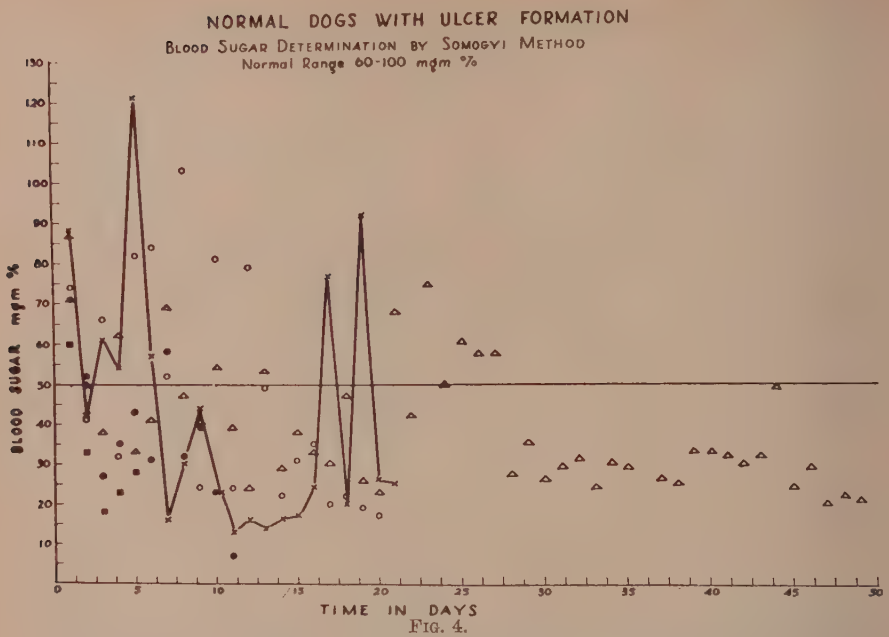

FIG. 4.

Scattergram of blood sugar concentrations of 5 normal dogs which had received commercial protamine zinc insulin in sufficient quantity to reduce the blood sugar level below 50 mg % and eventually result in the animal's death. A curve is drawn through one set of points. Each of these animals developed peptic ulcers.

of the stomach and duodenum(6). This factor is believed to be formed by the alpha cells of the islets of the pancreas while insulin is produced by the beta cells. Islet cell adenomata consist largely of beta cells. Can it be that insulin free of the H-G factor will fail to favor the formation of ulcers? This question is being studied by repeating the above work using purified insulin. Is the H-G factor responsible for peptic ulceration through its glycogenolytic property, or is ulceration due primarily to increased gastric activity induced by hypoglycemia?

Summary. 1. Dogs were maintained in a state of hypoglycemia by the administration of insulin. 2. These animals were divided into 2 equal groups. The animals of one group were pancreatectomized. 3. Half the animals of each group developed ulcers. 4. The absence of peptic ulcer formation in patients suffering from islet cell tumors and hypoglycemia is discussed.

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Virus Interference by Serially Passed Hodgkin's Disease Extracts in Chicken Eggs.* (17958)

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Many workers have joined in the search for an etiologic agent in Hodgkin's Disease (H.D.) and many biological and chemical technics have been tried without solving the problem. An infectious agent has long been suspected as being concerned in the process, and ever since Gordon's(1) report, repeated suggestions of a possible virus etiology have appeared; however, no significant or confirmed evidence of its presence has been reported.

Extensive studies of H.D. by the use of the fertile chicken egg has been made by Bostick(2) in efforts to detect any distinguishing characteristic that might be present. A slight lethal effect of serially passed H.D. extracts on chicken eggs was noted; so the experiments were continued in order to detect other distinguishing characteristics.

Experimental procedures. Fresh H.D. lymph nodes were ground with sterile sand, filtered through a Seitz filter if contaminated, and inoculated into the amniotic sac of 7 to 20 fertile chicken eggs, which had been incubated at 37.5°C for 7 days. After inoculation they were incubated at 35.5°C. The amniotic fluids (Am. F) of some live eggs and all of those dying between 2 and 9 days later were harvested and pooled. After culturing both aerobically and anaerobically, these pooled fluids were inoculated into another series of 7 to 20 eggs. If bacterial contamination occurred, the fluid was Seitz filtered before being passed on. All material was filtered on at least one occasion and had been serially passed through at least 4 (and usually 10) groups of eggs before being used in any interference experiments. Identical but separate serial passages were carried on with non-H.D. cancer-laden lymph node extracts, and this was used as control material. These control tissues were obtained from 4 separate patients. The H.D. lymph nodes were originally obtained from 9 separate patients. They were carried separately in serial passages and pooled with each other from time to time as indicated in the interference technics below.

Interference technics. Groups of 8 to 20 fertile eggs were inoculated with 0.1 cc of Am. F. in the manner described above. One group received Am. F. from control serial passages and the other from H.D. serial passages. After 72 hours incubation, all of the eggs were inoculated into the amniotic sac with 0.02 cc of Lee virus having a hemagglutination titre of 5 units. The Lee virus used was an egg adapted strain that had been transferred in the amniotic sac at intervals over many months. For the test, the Lee Am. F. was harvested 24 hours after inoculation and its hemagglutination titre was determined by the method of Salk(3). The fluids with a titre of 1:10,240 or over were pooled, retitred and then frozen in the ice box at −18°C until used. Usually the Lee was stored in separate vials, and only one of them at a time was thawed and used as needed. After the inoculation of the Lee virus the eggs were incubated at 35.5°C for 18 hours, whereupon all of the live eggs were placed in the ice box at 8°C for 3 hours. Then each was opened, the Am. F. cleanly pipetted off and a smear made to demonstrate the absence of bacterial contaminants. The Am. F. of each egg was tested for the amount of Lee virus present, as demonstrated by its hemagglutination titre.

Results. The technic described above is the result of many months of experimental probing. Before it was evolved the experiments

* This research was supported in part by a grant-in-aid from the National Institute of Cancer, United States Public Health Service, and in part by a gift from Mr. and Mrs. Frank Schwabacher.

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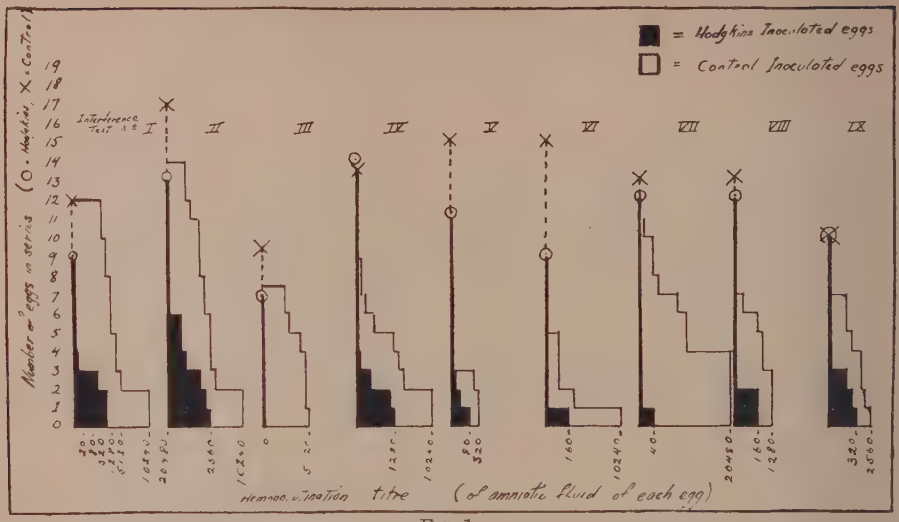

FIG. 1.

Contrast of influenza interference effects by Hodgkin's disease and control amniotic fluid as shown by comparison of their respective hemagglutinative titres.

did not show interference. However, after it was established, essentially all H.D. Am. F. showed virus interference capacity. The H.D. material was first tested for interference activity by using pooled Am. F. derived from 9 patients. When that revealed interference the tests were made on pooled Am. F. from 3 patients, then from 2 patients, and finally on the Am. F. derived from single H.D. cases. The results of the tests are indicated in Fig. 1.

Discussion. The capacity of Am. F. from H.D. inoculated eggs to interfere with the growth of Lee virus in fertile eggs was demonstrated repeatedly. This characteristic was possessed by material from multiple cases of H.D. The factor concerned must possess the capacity to reproduce in fertile chicken eggs since it was present in Am. F. after more than 10 serial passes in chicken eggs. By the 10th serial passage many months had passed since the original inoculation, and the dilution of the original H.D. tissue extract was at least 10^{-20}. Also the factor is Seitz filterable, since all Am. F.s were so filtered on at least one occasion, and usually on several, before being used in these experiments.

All of the non-H.D. control material was treated exactly like all H.D. material, and it represented the extracts of lymph nodes from 4 separate patients. Duplicate experiments designed to demonstrate that a parallelism existed between the hemagglutination titres and the infectivity titres of the Lee virus were not done at this phase of the study. Since interference activity was demonstrated in all H.D. Am. F. tested, it seems most unlikely that these results could be explained on the basis of having picked up a vicarious virus that perchance existed in human tissues. It may well be that this characteristic of H.D. extracts may be of greatest importance, for at last a difference between H.D. and other tissues has been demonstrated. Numerous obvious technics of biochemical, serological and immunological type can be focused on the problem so as to directly give, if possible, this factor etiologic significance. Similar technics, when applied to studies in other types of malignant lymphomas and reticuloendotheliosis, might be most revealing in an etiological sense.

Conclusions. The Am. F. harvested from series of fertile chicken eggs originally inoculated with H.D. tissue extracts showed virus interference capacities. Upon inoculation into fertile chicken eggs they were able to interfere with or to completely inhibit the growth of Lee virus subsequently inoculated into these

eggs. This factor was encountered in all H.D. Am. F.s tested and may be of etiologic significance in the disease. This factor is filterable, doubtless has the capacity to reproduce itself and probably has some slight lethal effect on chicken embryos.

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Neutralization of Pit Viper Venom by King Snake Serum.* (17959)

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There is considerable published evidence that certain venomous and non-venomous animals show some degree of tolerance to animal venoms(1-4). Numerous authentic incidents are recorded in the literature of snakes being bitten by other venomous snakes resulting in some instances in death and sometimes in survival(5). The fact that death may occur indicates that tolerance is limited; but, when the approximate amount of venom injected by the strike is considered on the basis of dosage per unit of body weight, the tolerated dose in the snake must be very large in comparison with that in man in order to permit any survivals. It has also been shown experimentally that some homologous and non-homologous serums exert a limited antivenin action(1,6,7).

The present work was suggested by the common belief in rural areas of this region that the king snake is immune to the bite of American vipers and by the report of Ditmars(8) of the survival of a king snake *(Lampropeltis getulus)* bitten by a water moccasin of much larger size. The investigation involved primarily a study of the degree of neutralization of viper venom by king snake serum as determined by the lethal action of the venom in mice. Experiments are also presented showing the low degree of toxicity of heat-detoxified king snake serum. With the technic used in our experiments it has been possible to show a higher degree of protection than, to our knowledge, has hitherto been reported for reptilian sera. An abstract of this work has already appeared (9).

Materials and methods. The chain king snake *(Lampropeltis getulus* Floridans) and the spotted king snake *(Lampropeltis getulus* Holbroki) obtained from Ross Allen's Institute, Fla. and from local collectors were used in these experiments. Blood was collected by severing portions of the tails following which it was allowed to clot and the serum separated by centrifugation. The serum was detoxified by heating it in test tubes in a water bath at 56°C for 30 minutes,

*. Supported in part by a special research grant from School of Medicine, Tulane University.

† Now situated at The Methodist Hospital, Brooklyn, N. Y.

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a process used for detoxication of snake serums by other workers(1,6,7). When not used immediately, it was stored at -20°C. The venoms of the Florida rattlesnake *(Crotalus adamanteus)* and of the water moccasin *(Agkistrodon piscivorus)* were obtained from Ross Allen's Reptile Institute in dry powdered form. Stock solutions of 1.0 or 0.5% were prepared in 0.9% NaCl solution and when not used immediately were preserved at -20°C. Wyeth's Antivenin *(Nearctic crotalidae)* Polyvalent was obtained on the open market.

Experimental procedure and results. a. *Neutralization of moccasin venom by king snake serum.* The toxicity of moccasin venom was determined by intraperitoneal injection into 97 white mice of varying dosages of from 3 to 15 mg/kg in volumes of from .13 to .45 cc. The LD_{50} as evaluated by the method of Miller and Tainter(10) was 6.25 ±s.e. .34 mg/kg. Most deaths occurred within 12 hours after injection, rarely after 24 hours, and in only 1 case after 48 hours. The volume of detoxified king snake serum which would save mice from an arbitrary dosage of 15 mg/kg (approximately 2½ LD_{50}'s) was then determined. Stock solutions of venom were diluted with varying proportions of 0.9% NaCl solution and serum to yield a venom concentration of 0.1%. After standing for 15 minutes, doses of the mixture containing 15 mg/kg of venom and from .37 to 3.0 cc/kg of serum were injected intraperitoneally into 114 mice. Sixty percent of the mice receiving 1.0 cc/kg of king snake serum were saved from 2½ LD_{50}'s of moccasin venom. Treatment of all data showed an ED_{50} of 1.0 ± s.e. 0.1 cc of serum per kg of mouse. This dosage of venom without serum caused death in 22 mice in from 20 minutes to 2 hours, only 3 surviving longer than 1 hour. In 53 mice essentially the same results were obtained by the intraperitoneal injection of 15 mg/kg of venom, followed immediately by king snake serum, without mixing *in vitro*. Half of the mice were saved by even less than 1 cc/kg of serum (ED_{50} = .71 ± s.e. .12 cc/kg). In an experiment in which 47 mice were used approximately 3 cc/kg of Wyeth's polyvalent antivenin was necessary to achieve the same effect (ED_{50} = 3.15 ± s.e. .49 cc/kg).

TABLE I.
Antidotal Action of 1 cc of Detoxicated King Snake Serum Against Moccasin Venom in Mice.

Venom, mg/kg	No. of mice	
	Used	Survived
33	8	8
45	5	4
45*	6	5
60*	4	0
67.5	5	1
100	4	0

* Venom and then serum injected separately (intraperitoneally). Others mixed before inj. Wt range of mice, 24-35 g.

By both technics a total dosage of approximately .1 and .03 cc of king snake serum protected practically all mice and about half of the mice, respectively, from 2½ lethal doses of venom. Since these amounts of serum were far below the range of toxic dosage, the protective action of a much larger but still non-toxic dose of serum was evaluated. The results are presented in Table I.

All mice were protected against approximately 5 LD_{50}'s (33 mg/kg) of venom by 1 cc of serum per mouse, while 9 of 11 mice were protected against approximately 7 LD_{50}'s (45 mg/kg).

b. *Neutralization of rattlesnake venom by king snake serum.* Rattlesnake venom proved to be more toxic in mice by intraperitoneal injection than moccasin venom. The LD_{50} determined on 50 mice was evaluated at 1.7 ± s.e. .1 mg/kg of body weight. When the administration of 4.0 mg/kg of venom (approximately 2½ LD_{50}'s) was followed immediately by detoxicated king snake serum, both by intraperitoneal injection, .4 cc/kg of serum saved approximately half the mice while twice this dose saved 93.5% of the mice. The latter dose of Wyeth's polyvalent antivenin, however, saved only 8 of 30 mice. The results are presented in Table II.

c. *Toxicity of king snake serum.* Of equal interest to the antidotal action of detoxicated king snake serum against venoms is the relatively low toxicity of the serum itself.

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TABLE II.
Antidotal Action of Detoxicated King Snake Serum and Wyeth's Antivenin Against 2½ LD_{50}'s of Rattlesnake Venom in Mice.
Separate intraperitoneal injections.

King snake serum, cc/kg	Mice saved	
	No.	%
.4	14/30	47
.8	29/31	93
1.3	5/5	100
Antivenin		
.8	8/30	27
1.3	5/5	100

The untreated serum is definitely toxic. Its lethal dose was not determined by us, but in a few mice 1 cc injected intraperitoneally produced death within 2 hours, and .25 cc by this route has been reported as a surely lethal dose(7). It is also well known that the toxicity is decreased by heating(1,6,7). The few experiments described here show that the antidotal dosages of the detoxicated serum are well below the range of toxicity. The largest antidotal dosage used was 1 cc (approximately 30 cc/kg) in mice weighing from 24 to 35 g. This dosage, injected intraperitoneally into 7 normal mice, produced no observable effect. A much larger dosage of detoxicated serum (150 cc/kg—total dosages of 2.25-4.35 cc) was tolerated intraperitoneally by 5 mice without incident. The following intravenous doses were administered to other animals without obvious effect: guinea pig (wt. 339 g) 5 cc; rabbit (wt. 1.55 kg) 5 cc; dog (wt. 13 kg) 20 cc. An intravenous injection of 16 cc of the detoxicated serum over a period of 13 minutes was well tolerated by one of us (V.P.).

Discussion. It is evident from previous isolated observations(8) and from the results reported here that detoxicated king snake serum contains a factor with high neutralizing potency against moccasin and rattlesnake venoms. We are unable to explain why Rosenfeld and Glass(7) were unable to demonstrate protection against the lethal effects of venoms by the serum of this snake although they observed an inhibition of the hemorrhagic action. A high degree of natural immunity of the king snake was incidentally confirmed by the intramuscular injection into one snake weighing 470 g of 140 mg of moccasin venom in 4 doses within 5 days. A second snake weighing 770 g similarly received 1035 mg of venom in 5 doses within 21 days, the largest single injection being 320 mg. The only adverse effect observed was slight swelling at the site of injection with the larger doses. Such doses are of a definitely higher order than lethal doses in higher animals and in man. Natural immunity is, of course, not confined to the king snake as has been shown by the authors cited above. In this connection we were able to demonstrate neutralization of moccasin venom by serums of the blue racer snake and water snake which was of the same general order as that by king snake serum. A possible therapeutic value of king snake serum can only be determined by further investigation of its potency by other technics and on other animals and by more extensive testing of its toxicity, but the results obtained in these experiments are suggestive in this regard. From the standpoint of producing the serum, an adequate number of king snakes could probably be obtained as easily as the rattlesnakes which serve as a source of venom for the immunization of horses over a period of several months in the preparation of therapeutic antivenins.

Summary. King snake serum, detoxicated by heating, and in doses well below toxic levels protected white mice against as high as 7 LD_{50}'s of moccasin venom injected intraperitoneally, when serum and venom were mixed *in vitro* or injected separately A protective action against rattlesnake venom was also demonstrated. Wyeth's polyvalent antivenin used by the same technic showed a definitely lower degree of neutralizing potency against both moccasin and rattlesnake venoms.

Dr. Max Littman and Dr. Foster N. Martin, Jr., have contributed many useful suggestions and criticisms. Mr. Bennett Elisberg informed us of the technic of collecting blood from the tail of the snake. Some of the mice were contributed by Frank and Jimmy Pittari, Phyllis Warden and Joy Daray.

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Loss of Insulin Hypersensitivity and Development of Diabetes in Hypophysectomized Dogs Produced by Purified Growth Hormone.*† (17960)

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It is well established that removal of the anterior pituitary gland renders the animal highly sensitive to the hypoglycemic action of insulin. Previous work from this laboratory(1) has shown that the hypersensitivity develops gradually following removal of the hypophysis, reaching its maximum in 5 to 6 weeks. Since the adrenal and thyroid glands undergo gradual atrophy when deprived of their hypophyseal tropic hormonal regulation, the role of these endocrine organs in insulin hypersensitivity was studied. Both thyroxin (2) and adrenal cortical extract(3) were found to antagonize the action of insulin. When very large amounts of a potent adrenal cortical extract were administered a marked anti-insulin action was noted. Nevertheless, it could not abolish the hypersensitivity. The present report deals with the effect of purified anterior pituitary growth hormone on the insulin response and the glucose tolerance of hypophysectomized dogs.

Method. This study was carried out on trained, unanesthetized normal and hypophysectomized dogs.§ Only those hypophysectomized animals were used that exhibited maximal insulin hypersensitivity. All insulin and glucose tolerance tests were started 17 to 18 hours after the last feeding. Unless otherwise stated, 0.025 unit of insulin (Lilly)‖ per kg body weight (the test dose) was given intravenously and the blood sugar changes followed for a 4 hour period. The response to intravenous glucose, 0.075 g/kg/min. for 10 min., was also followed for 4 hours. The growth hormone used was prepared by Armour and Co. (Lot No. 22KR1)¶ In the experiments to be reported the first dose of the hormone was always given intramuscularly in the afternoon and followed immediately by feeding. The second dose was given intravenously in the fasting state, 17 to 18 hours later. Thereafter, the hormone was administered intramuscularly. The daily dose of growth hormone was always 1 mg/kg body weight.

Results. A total of 68 insulin experiments were performed on normal and hypophysectomized dogs before and during growth hormone administration. In Table I we have recorded the results of representative experiments. The administration of the test dose of insulin to normal dogs produced no change in blood sugar or only a slight hypoglycemia of short duration. The response of normal dogs as recorded in Table I is a composite of the values obtained in 18 animals. The test dose of insulin in the hypophysectomized dog Hy9, as in all our hypophysectomized animals, produced a very marked hypoglycemia. Normal animals require 60 to 100 times the test dose to manifest a comparable hypoglycemic response(1). Two doses of growth hormone markedly diminished the hypoglycemic response to insulin in all the hypophysectomized dogs. After four or more doses the exag-

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† This work was presented before the American Physiological Society at Atlantic City, April, 1950, and appeared in the *Fed. Proc. Am. Soc. Exp. Biol.*, 9, 30, 1950.

‡ Dazian Foundation Fellow.

1. Slater, I. H., De Bodo, R. C., Weisberg, H. F., and Kiang, S. P., *Fed. Proc.*, 1948, v7, 116.

2. Unpublished observations.

3. de Bodo, R. C., Kiang, S. P., and Slater, I. H., *Fed. Proc.*, 1949, v8, 32.

§ We would like to thank Dr. A. C. Bratton, Jr., of Parke, Davis and Co. for supplying the Topical Thrombin which was most helpful in controlling hemorrhage during the operation.

‖ Insulin was kindly supplied by Dr. K. K. Chen of Eli Lilly and Co.

¶ We are greatly indebted to Drs. E. E. Hays and I. M. Bunding of Armour and Co. for their generous supply of growth hormone.

TABLE I.
Insulin Sensitivity and Glucose Tolerance Tests in Normal and in Hypophysectomized Dogs Before and During Growth Hormone (GH) Treatment.

	(A) Insulin sensitivity tests					(B) Glucose tolerance tests			
	Normal	Hy9				Normal	Hy9		
	Doses of GH*					Doses of GH*			
	0	0	2	9		0	0	14	
Time in min.	Blood sugar in mg %				Time in min.	Blood sugar in mg %			
0	77	63	73	102	0	79	61	110	
	Insulin .025 u/kg intrav. at 0 time					Glucose .075 g/kg/min. intrav. for 10 min.			
10	67	49	64	100	10	261	272	332	
20	66	35	55	96	15	205		314	
30	74	24	54	94	40	104	127	264	
40	75	29	54	92	50		86		
50	77	31	53	92	55	85		256	
60	77	29	49	92	60		54		
75		32	54	95	70	82		246	
90		35	53	92	85	83	57	236	
120		34	55	103	100	80	52	220	
150		38	59	108	115	80		204	
180		39	60	103	145	80		174	
210		40	60	104	160	79	50	162	
240		40	59	103	190		†	146	
					220		†	130	
					250		†	117	

* Dose of growth hormone: 1 mg/kg body wt.
† Convulsions.

gerated response to insulin disappeared. In fact, after prolonged administration of growth hormone the hypoglycemic response in the hypophysectomized animal following 1.5 units of insulin per kg was less than that in normal animals.

Representative results of 28 intravenous glucose tolerance tests in normal and hypophysectomized dogs before and during growth hormone administration are also recorded in Table I. The untreated hypophysectomized dog responds to intravenous glucose like the normal animal, except for a characteristic secondary hypoglycemic phase. The growth hormone treated hypophysectomized dogs displayed the type of response to intravenous glucose infusion which occurs in diabetic animals, *i.e.*, the blood sugar rose to higher levels and the return to the fasting level was very much delayed. It is noteworthy that the secondary hypoglycemic phase disappeared. An impaired glucose tolerance was observed as early as after 2 doses of growth hormone. Continued administration of the hormone resulted in progressive impairment of the glucose tolerance. A further indication of the diabetogenic action of purified growth hormone was the progressive elevation of the fasting blood sugar noted after daily hormone administration (Table I). The elevation of the fasting blood sugar level is more apparent when compared to the low fasting blood sugar level of untreated hypophysectomized dogs. We did not note either glycosuria or acetonuria in hypophysectomized animals during the growth hormone treatment.

While this work was in progress Young and his colleagues(4) reported the production of glycosuria in normal cats with purified growth hormone. Quite recently Houssay and Anderson(5) obtained diabetes in partially depancreatized dogs and cats treated with large doses of growth hormone.

Discussion. It is evident from our experiments that the marked insulin hypersensitivity of the hypophysectomized dogs was completely abolished by the administration

4. Cotes, P. M., Reid, E., and Young, F. G., *Nature*, 1949, v164, 209.

5. Houssay, B. A., and Anderson, E., *Endocrinology*, 1949, v45, 627.

of purified growth hormone. When this occurred an impairment of glucose tolerance, *i.e.*, a diabetic state, was also present. Evans and his colleagues(6) have shown that growth hormone produces nitrogen retention and lowers the blood amino nitrogen. In our experiments, therefore, increased gluconeogenesis, as a factor accounting for the loss of hypersensitivity and the diabetic state, can be minimized. The alteration in carbohydrate metabolism effected by growth hormone must be attributed to decreased carbohydrate utilization. That peripheral utilization of carbohydrate is diminished by purified growth hormone, is supported by the work of Stadie *et al.*(7) and Park *et al.*(8). These investigators found a decrease in carbohydrate utilization in the isolated diaphragm of growth hormone treated rats. It is apparent from our work that a physiological antagonism exists between growth hormone and insulin. In view of this latter phenomenon, the effects of growth hormone administration to intact animals are in all likelihood greatly modified by compensatory adjustment of the insulin-secreting cells of the pancreas. If the islets of Langerhans of growth hormone treated animals had been able to adequately adjust themselves to the peripheral inhibition of carbohydrate utilization by increasing their insulin output, no impairment of glucose tolerance would have been observed.

It is our opinion that in the hypophysectomized animal the absence of endogenous growth hormone is an important factor in causing the insulin hypersensitivity. If we had succeeded in obtaining physiological levels by hormonal replacement therapy, we certainly should not have observed a diabetic state. We consider the impairment of glucose tolerance induced by growth hormone to be the result of overdosage, an exaggerated manifestation of a physiological process.

Summary. The insulin hypersensitivity characteristic of hypophysectomized dogs was completely abolished by purified anterior pituitary growth hormone. Concomitantly an impairment of glucose tolerance, *i.e.*, a diabetic state, was produced.

6. For references see Li, C. H., and Evans, A. M., Recent Progress in Hormone Research, Academic Press, Inc., New York, 1948, v23, pp. 3-44.

7. Stadie, W. C., Haugaard, N., Hills, A. G., and Marsh, J. B., *Am. J. M. Sc.*, 1949, v218, 275.

8. Park, C. R., and Krahl, M. E., *J. Biol. Chem.*, 1949, v181, 247

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Effects of B-Diethylaminoethyl Xanthene 9-Carboxylate Methobromide (Banthine*) on Human Gastrointestinal Function.† (17961)

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(Introduced by C. M. Carpenter)

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Medical therapy in peptic ulcer has relied to a considerable extent upon agents that will neutralize or buffer the acid gastric juice which appears to be so important in the production of peptic ulcer. In searching for medication to prevent or diminish the production of acid gastric secretion, the belladonna derivatives, notably atropine, have been used because of their effect as para-sympathetic antagonists. The usefulness of this group of drugs has been limited because depression of gastric secretion is not affected unless other physiological side effects are produced(1,3). There are also unpredictable individual tol-

* Banthine furnished by courtesy of G. D. Searle Co., Chicago, Ill.

† Reviewed in the Veterans Administration and published with the approval of the Chief Medical Director. The statements and conclusions published by the authors are the result of their own study and do not necessarily reflect the opinion or policy of the Veterans Administration.

erances(3). Atropine has been found to depress the volume of gastric secretion but the acidity is not affected until the volume has been reduced to 40-60%(2). B-Diethylaminoethyl xanthene 9-carboxylate methobromide (Banthine) has recently been therapeutically employed largely as a depressant of gastric secretion. This drug is a quaternary ammonium compound and may be classed as an anti-cholinergic drug. In moderate dosage it seems to have a muscarine effect, blocking the post-ganglionic parasympathetic nerve endings. In large doses a nicotine or curariform response is noted(4).

Procedures. The studies reported here have been concerned with 3 phases of gastrointestinal function: (1) gastric secretion, (2) gastric motility, and (3) small intestinal motility. Gastric secretion has been studied in the fasting state, at least 12 hours following the last meal. Eight patients had duodenal ulcers and 2 had no demonstrable gastrointestinal disease having been admitted to the hospital for other unrelated conditions. Continuous gastric aspiration was performed and samples divided into 15 minute fractions. The volume of the specimen was measured and the acid values were determined by titration with 0.1 N sodium hydroxide within a few minutes of aspiration. When a control period of a reasonably constant secretory level had been obtained, 100 mg of Banthine was given by mouth with 30 cc of water. Aspiration was discontinued for 30 minutes and then resumed in the manner described for the control period. No gastric secretagogues were used in this study. Gastric motility was recorded on a kymograph by means of an intra-gastric balloon containing from 100-150 cc of air connected to a water manometer and an ink-writing tambour. Following a control period of 30-45 minutes 100 mg of Banthine with 30 cc of water was administered by mouth, and

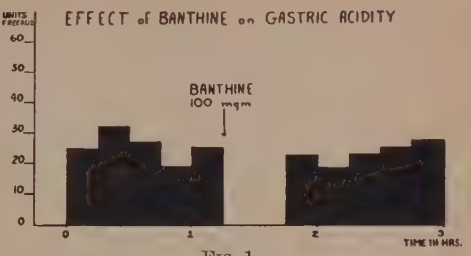

FIG. 1.

Composite chart of acidity values obtained in 10 patients. Following administration of Banthine, aspiration was discontinued for 30 min. and then resumed.

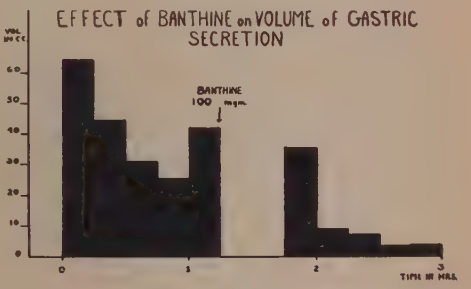

FIG. 2.

Composite chart of volume of gastric secretion obtained in 10 patients. Aspiration of the gastric contents was withheld for 30 min. following the oral administration of Banthine.

the recording continued. Small intestinal motility was recorded in a similar manner but a balloon containing 20 cc of air was used, being guided into the upper jejunum under fluoroscopic control. Gastric motility studies were made on 2 patients, both of whom had duodenal ulcers. Small intestinal motility records were obtained in 2 patients with regional enteritis.

Results. 1. Gastric secretion. 100 mg of Banthine diminished the volume of gastric secretion but produced no significant change in the titratable gastric acidity. The depression in volume occurred within 30-45 minutes following oral administration and persisted from 1.5 to 4 hours. In this small series no detectable difference in response was noted between the 2 control cases and the 8 ulcer patients. Fig. 1, a composite chart of gastric free acidity determinations obtained in the 10 patients before and after giving Banthine, shows little difference in the two periods. Fig. 2, a composite chart of gastric secretory

1. Atkinson, A. J. and Ivy, A. C., *Am. J. Dig., Dis.* 1937-38, v4, 811.

2. Keeton, R. W., Luckhardt, A. B. and Koch, F. C., *Am. J. Physiol.,* 1920, v51, 469.

3. Palmer, W. L., *J. Am. Med. Assn.,* 1933, v101, 1604.

4. Longino, F. H., Grimson, K. S., Chittum, J. R. and Metcalf, B. H., *Gastroenterology,* 1950, v14, 301.

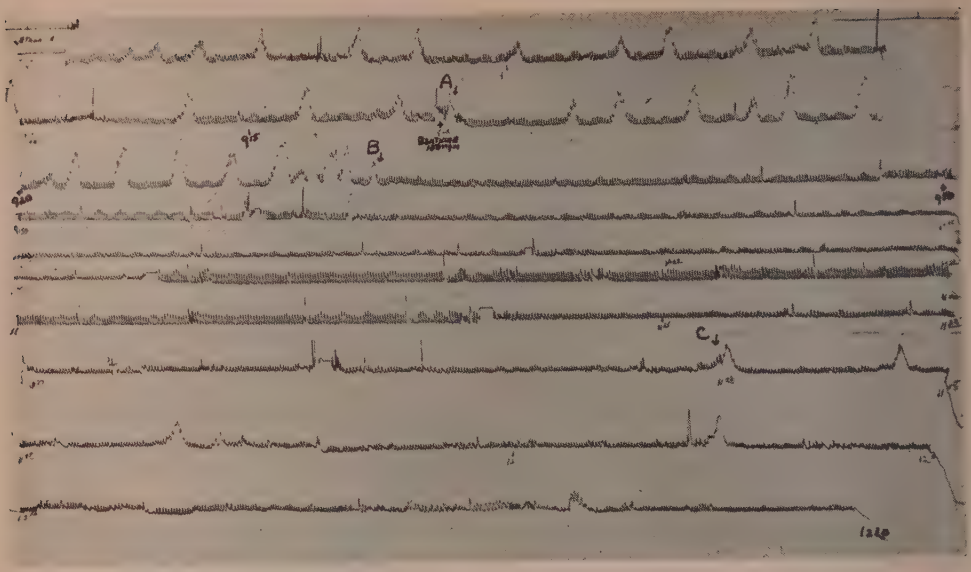

FIG. 3.

Gastric motility tracing obtained in patient with duodenal ulcer. 100 mg Banthine given orally at A. Gastric contractions were absent from B to C (approx. 2 hr). One line on the graph represents 20 min.

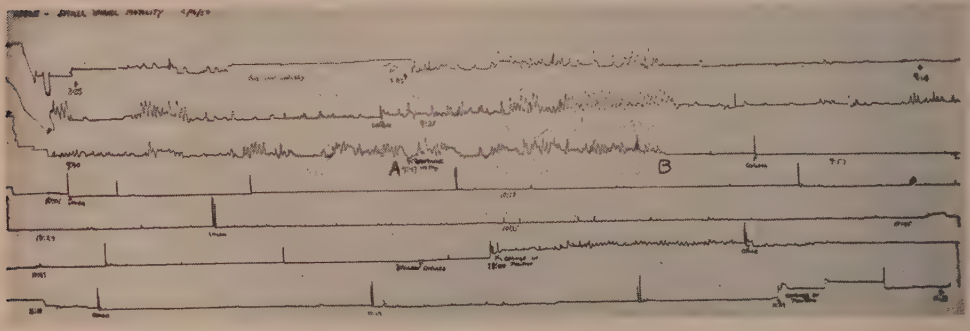

FIG. 4.

Tracing of upper jejunum motility obtained in patient with regional enteritis. Banthine (100 mg) administered at A with prompt cessation of contractions at B. One line on the graph represents 20 min.

volume, demonstrates a marked fall in the volume of secretion after Banthine.

2. Gastric motility. Banthine markedly reduced gastric motility, Fig. 3. Within 20-30 minutes following 100 mg orally, gastric contractions ceased and remained absent for approximately 2 hours. In one patient secretion and motility were recorded simultaneously and appearance of the depression of volume and motor activity were found to occur at the same time.

3. Small intestinal motility. In both patients in whom small intestinal motility records were obtained, marked depression of motor activity was found. Changes were apparent within 15 minutes after oral administration of the drug and persisted for as long as 2 hours, Fig. 4.

Summary. In 10 patients Banthine decreased the volume of gastric secretion without significantly altering gastric acidity. Gastric and small intestinal motor activity

was markedly diminished in 2 studies. Banthine depressed gastric secretion in a manner similar to atropine, but side effects were less pronounced. 100 mg of Banthine produced no undesirable side effects other than dryness of the mouth.

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Effect of Cortisone and Adrenocorticotropin Therapy on Serum Proteins in Disseminated Lupus Erythematosus. (17962)

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The serum proteins in patients with *disseminated lupus erythematosus* have been investigated electrophoretically. They appear to have a characteristic protein pattern. About 800 sera from patients with a great variety of diseases have been examined in our laboratory, and except for an occasional specimen from an unrelated condition, we have found no consistent duplication of this particular protein pattern (Fig. 1).

The serum was diluted with barbiturate buffer of pH 8.6 and an ionic strength of 0.1, and dialyzed either with mechanical stirring at room temperature(1), or without stirring for 48-72 hours in the refrigerator. An Amino-Stern electrophoresis apparatus equipped with the cylinder lens-diagonal diaphragm optical system of Philpot-Svensson was employed. Both the ascending and descending boundaries were recorded, but for the sake of simplicity, only the ascending boundaries are shown (Fig. 1 and Table I). While the total protein content of the sera is generally within normal limits(2), the albumin concentration is decreased; the α_2- and γ-globulins are increased, but the α_1 and β-globulin fractions remain in the normal range. This causes a reversal of the A/G ratio.

The γ-globulin may be increased to 50% of the total protein content. Hyper-γ-globulinemia is usually encountered either in infectious diseases or in some involvement of the liver *e.g.* cirrhosis. As the condition of the patient improves temporarily either under treatment or during a spontaneous remission of the disease, the amount of γ-globu-

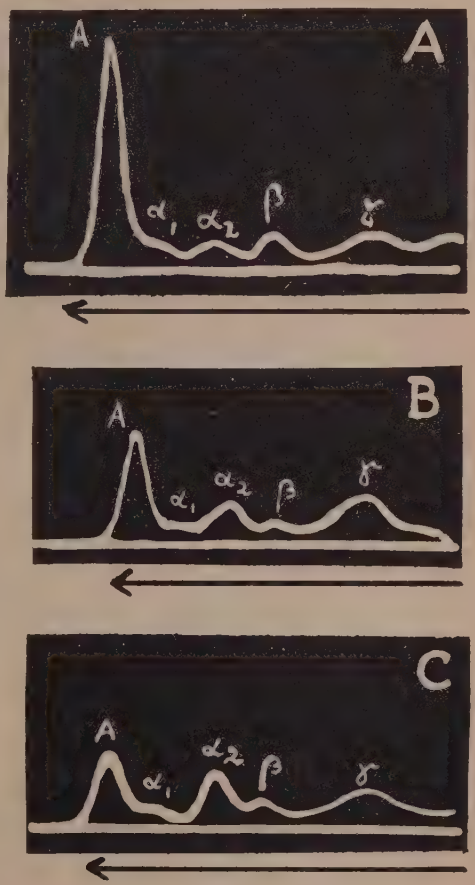

FIG. 1.

Representative electrophoretic patterns of normal serum (A), and of 2 sera from patients with *disseminated lupus erythematosus* (B, C).

1. Reiner, M. and Fenichel, R. L., *Science,* 1948, v108, 164.

2. Reiner, M., Fenichel, R. L., and Stern, K. G., *Acta Hematol.,* 1950, v3, 202.

TABLE I.
Electrophoretic Distribution of Serum Proteins in *Disseminated Lupus Erythematosus.*

No. of cases	Total protein (g %)	Albumin globulin A/G ratio	Relative % of total protein				
			Albumin	Globulins			
				Alpha$_1$	Alpha$_2$	Beta	Gamma
Normal(2) (Avg of 60)	7.22 ± 0.48	1.3	56.8 ± 3.0	7.2 ± 1.2	8.7 ± 1.5	12.8 ± 2.3	14.4 ± 2.4
Dis. lup. erythem. (20 cases) untreated	6.90 ± 0.27	0.5	33.4 ± 1.5	7.3 ± 0.3	15.4 ± 1.2	12.8 ± 1.0	31.1 ± 1.8

TABLE II.
Electrophoretic Distribution of Serum Proteins in Disseminated Lupus Erythematosus Before and After Cortisone and ACTH Therapy.

No. of cases	Total protein (g %)	Albumin globulin A/G ratio	Relative % of total protein				
			Albumin	Globulins			
				Alpha$_1$	Alpha$_2$	Beta	Gamma
5 patients (Before treatment)	7.07 ± .32	.6	34.7 ± 3.9	8.1 ± .3	12.5 ± 0.9	15.1 ± 2.0	29.6 ± 3.2
5 patients (After treatment)	6.11 ± .48	.8	43.9 ± 1.0	7.2 ± .6	12.7 ± 1.5	16.6 ± 0.8	19.5 ± 2.3

lin will decrease and the albumin will increase.

It is noteworthy that while the α_1 protein concentration is normal, the α_2-globulin content is increased to twice the normal amount. This fraction, during remission or after treatment, very rarely returns within normal limits.

In a study of the effect of adrenocorticotropin (ACTH) administration in two healthy male subjects, Sayers *et al.*(3) report that the serum protein content was unchanged. After one subject received 100 mg of ACTH, samples of blood were taken at 0, 3, 6, 12, 24, 48 and 96 hours. The second subject received 50 mg and samples were withdrawn at 0, 3, 6, 12 and 24 hours. The serum was equilibrated against veronal buffer at pH 8.6 and an ionic strength of 0.1. The electrophoretic distribution of the serum protein was normal and there was no significant change in either case following the injection of ACTH.

The sera of 5 cases of *disseminated lupus erythematosus* under therapy at Mount Sinai Hospital(4) at this time were examined before and after treatment with cortisone and ACTH for a period from 2 to 6 months. The average protein values are shown in Table II. It will be noted that following treatment, the albumin concentration increased from 34.7 to 43.9% while the γ-globulin decreased from 29.6 to 19.5%. The α_2-globulin remained remarkably constant at the elevated level of 12.7%. Further studies on this fraction are in progress.

Electrophoretic diagrams recorded in a typical case (M.F.) before and after treatment with a total of 2 g of cortisone and 3.5 g. ACTH over a 3 months period are reproduced in Fig. 2.

Coburn and Moore(5) have presented the

3. Sayers, G., Burns, T. W., Tyler, F. H., Jager, B. V., Schwartz, T. B., Smith, E., Samuels, L. T. and Davenport, H. W., *Jour. Clin. Endocrin.*, 1949, v9, 593.

4. Baehr, G., Soffer, L. J., Boas, N., Levitt, M., and Gabrilove, J. L., paper delivered at Ass. Am. Physicians, Atlantic City, May 1950 in press.

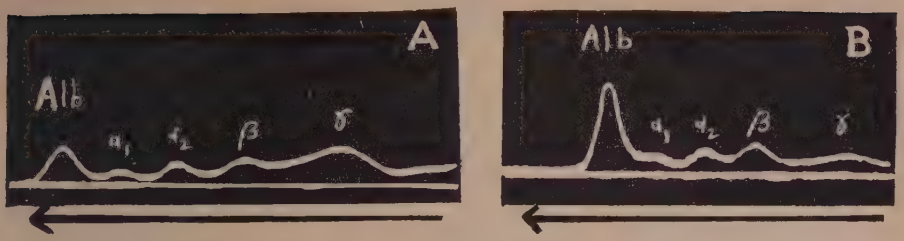

FIG. 2.

Electrophoretic diagrams of the serum proteins of one patient with *disseminated lupus erythematosus* before (A) and after therapy with cortisone and ACTH (B).

electrophoretic patterns obtained from the plasma of 2 cases of lupus erythematosus. Their data are somewhat difficult to interpret, since there seem to be some printing errors in their tables listing the protein composition of the plasma. Since phosphate buffer of pH 7.4 was used, they obtained no separation of the a_1-component from the albumin fraction. Due to turbidity they had difficulties in securing satisfactory diagrams.

In this study it was also found that occasionally the β- and γ-globulin boundaries were somewhat obscured, probably due to the presence of lipids or lipoproteins, even though the sera were not frankly lipemic. After high-speed centrifugation at low temperatures, a creamy layer rose to the top, leaving clear serum underneath, making it possible to obtain a well-differentiated electrophoretic diagram.

Summary. The electrophoretic distribution of the serum proteins of 20 patients with *disseminated lupus erythematosus* showed a lowered albumin content with a considerable increase in the a_2- as well as γ-globulin concentration. The sera of 5 patients were studied before therapy with cortisone and adrenocorticotropin (ACTH). After a clinical remission had been produced by these agents, it was found that the albumin and γ-globulin components progressed towards normal levels whereas the a_2-globulin fraction remained unchanged.

Thanks are due to Dr. George Baehr and the members of the First Medical Service for their cooperation throughout this study. The technical help of Sheldon S. Gilbert and Harold Corey is acknowledged with appreciation.

5. Coburn, A. F. and Moore, D. H., *Bull. Johns Hopkins Hosp.*, 1943, v73, 196.

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Achromotrichia Due to Copper Deficiency. (17963)

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A purified diet, similar to diets used in many other laboratories has been employed in this laboratory as a presumably adequate diet for the albino rat. Recently it was observed that black rats on this diet developed an achromotrichia which could be prevented by liver or yeast. Further investigation disclosed that the activity of these substances was due to copper. Achromotrichia due to copper deficiency has been described previously (1-6). In most of these studies the ach-

1. Kiel, H. L., and Nelson, V. E., *J. Biol. Chem.*, 1931, v93, 49.

2. Gortner, F. J., *Nature*, 1935, v136, 185.

3. Free, A., PROC. SOC. EXP. BIOL. AND MED., 1940, v44, 371.

romotrichia developed on diets composed principally of milk or other crude dietary materials. The present report will describe a type of achromotrichia responding to copper which was produced with a highly purified ration which permitted normal growth and hemoglobin production.

Methods. Black, Long-Evans, male rats from the National Institutes of Health colony were housed individually in raised, wire-bottom cages and placed on the diets when 21 to 29 days of age with weights of 25 to 59 g. Each group in individual experiments was balanced by litter and weight. The composition of the basal diet 1718B is indicated in Table I. The O&M salt mixture (7) and salt No. 550* were prepared in this laboratory following the original instructions. Copper analyses† were by the carbamate method(8). Hematocrits were determined as outlined by Van Allen(9). The degree of grayness was evaluated for each animal on an arbitrary 0 to 4 + scale. To facilitate the comparison of different groups of rats on the same or different diets the plus marks assigned to each rat in a group were totaled and divided by the number of rats in the group to give a Grayness Index. Thus, an index of 4 would represent maximum grayness in every rat. All of the Grayness Index values herein reported were calculated at the 8th experimental week.

TABLE I.
Composition of Diet 1718B.

	g
Casein (vit. free)	20.
Sucrose	71.7
Starch	1.
Wesson oil	3.
Salt O and M	4.
Cystine	.3
	mg
Thiamine	.2
Riboflavin	.3
Pyridoxine	.25
Ca pantothenate	2.
Choline chloride	100.
Inositol	10.
Nicotinic acid	2.
2-methylnaphthoquinone	.1
Biotin	.01
Folic acid	.1

Vit. A, D, and E as supplements.
A and D—2,000 and 400 U.S.P. units twice weekly.
E—3 mg tocopherol once each week.

Results. Development of gray hair. Rats maintained on the 1718B diet grew at a rate comparable to those on Purina Lab Chow, Table II, and seemed to develop normally except that within 5 to 6 weeks most of the animals developed marked graying of the hair. The first grayness was noted as early as 10 days and developed progressively reaching a maximum incidence and severity usually about 8 weeks after starting the diet. In about half of the rats the graying process appeared diffusely over the back. In others a

4. Henderson, L. M., McIntire, J. M., Waisman, H. A., and Elvehjem, C. A., *J. Nutrition*, 1942, v23, 47.

5. Smith, S. E., and Ellis, G. H., *Arch. Biochem.*, 1947, v15, 81.

6. Singer, L., and Davis, G. K., *Science*, 1950, v111, 472.

7. Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1917, v32, 369.

* Salt No. 550 differed from O&M salts in that it had only 1/10 as much NaF and contained .25% added $CuSO_4 \cdot 5H_2O$.

† We wish to thank Mr. William C. Alford and his staff of the Microanalytical Laboratory of this Institute, who did all of the copper analyses reported in this paper.

‡ *Substances Active*—Whole Dried Liver 5%; Dried Brewers Yeast 5%; Dried Alfalfa 20%; $CuSO_4 \cdot 5H_2O$ 0.005%; Purina Lab Chow. *Substances slightly active*—Wheat germ 10%; O&M salts 8%; Salts No. 550, 4%. *Substances inactive*—Wheat 20%; Corn Meal 20%; Soybean Meal 10%; Fish Meal 10%; Whole dried milk 10%; Lactalbumin 10%; Casein 40%; Cerelose 72%; Starch 72%; Crisco 20%; Tyrosine, Phenylalanine and Tryptophan 0.2% each; Cystine 0.3%; Carrots and Kale fed separately ad lib; $CoSO_4 \cdot 7H_2O$ or $MnSO_4 \cdot 4H_2O$ 0.02% each; $FeSO_4 \cdot 7H_2O$ 0.18%; Liver extract 3 U.S.P. units or B_{12} 2 μg each given daily subcutaneously; Pantothenic acid 10, Niacin 10, Thiamin 1, Pyridoxine 1, Riboflavin 2, p-amino benzoic acid 100, Choline 200, Ascorbic acid 200 mg % each used separately; double quantities of vitamins used together as in 1718B diet.

8. Sandell, E. B., "Colorimetric Determination of Traces of Metals", Interscience Publishers, Inc., New York, N. Y., 1944, 221.

9. Van Allen, C. M., *J. Lab. Clin. Med.*, 1925, v10, 1027.

TABLE II.
Effect of Various Diets on Growth and Achromotrichia.

Diet	No. of rats	Cu in diet, μg	Gain in body wt, g/wk*	No. of gray rats†	Grayness index
Purina Lab Chow	10	21.5	21.4	0	0
1718B	80	14.6	20.0	68	1.7
" + 5% liver powder	30	20.0	24.7	5	0.3
" + 10% liver powder	10	25.6	27.0	0	0‡
" + 5% brewers yeast	20	18.3	22.6	0	0
" + 10% brewers yeast	10	22.0	24.4	0	0‡
" + 20% dried alfalfa	10	21.0	26.3	3	0.4
" + .02% $CuSO_4 \cdot 5H_2O$	30	64.6	21.2	1	0.03
" + 10% dried wheat germ	10	—	24.2	4	0.6
" + 4% O & M salt	10	15.5	21.4	8	1.2

* Growth rate calculated for first 4 wk on diet only.
† After 8 wk on the diet.
‡ Observed 12 wk.

TABLE III.
Amount of Copper Necessary to Prevent Achromotrichia When Added to 1718B Diet.

No. rats	Cu added to diet, μ/g	Growth, g/wk	No. showing detectable graying	Grayness index
9	.25	20.5	5	1.6
9	2.5	22.8	2	.3
10	12.5	22.9	0	.0
10	25.	22.	1	.2
9	50.	20.2	1	.1

definite pattern developed with a gray strip down the middle of the back, a patch at the base of the tail or a collar patch across the back of the neck. These patterns gradually disappeared as the remainder of the hair grayed so that the end result in every animal was a rather uniform coat of various degrees of grayness. Eighty rats on this diet showed a Grayness Index of 1.7. About 20% of these showed no graying, or graying too slight to assess accurately. A few of the rats developed hair which was a dull red rather than gray. Most of the rats with marked grayness showed considerable thinning of the hair with loss of much of the fine undercoat. Diet 1718B was completely ineffective within periods of 12 weeks in producing gray hair in adult rats which had been reared on stock rations.

Prevention of gray hair. A large number of substances were tested for their ability to prevent the achromotrichia‡ (Table II) by adding appropriate amounts to the 1718B diet at the expense of sucrose. Whole dried liver, 5%, or dried brewer's yeast, 5%, were quite effective. Large amounts of all the water soluble vitamins were without effect, singly or in combination. Pantothenic acid and p-aminobenzoic acid, which have been implicated in other types of achromotrichia, were completely inactive. When diet 1718B was supplemented with copper in amounts as small as 2.5 μg per g in the form of $CuSO_4 \cdot 5H_2O$, gray hair was largely prevented. Larger amounts were somewhat more effective (Table III).

Copper content of diets. It seems evident that 4% of O&M salt mixture supplied an insufficient amount of copper. Even when this salt mixture was increased to 8 percent, achromotrichia still developed (Table II). This mixture contained no copper added as such but depended on the other ingredients to supply trace metals as impurities. Analysis of this salt mixture showed 23 μg of copper per g which would supply less than 1 μg of copper per g to the whole 1718B diet. Analysis of the diet, however, showed 14.6 μg of copper per g indicating that other ingredients in diet 1718B supplied considerable

TABLE IV.
Effect of Ashing on Achromotrichia Preventing Effect of Liver Yeast and Alfalfa.

No. rats	Diet	No. of gray rats	Grayness index
10	1718B	8	2.2
8	" + liver ash	1	.12
10	" + alfalfa ash	3	.3
10	" + yeast ash	3	.6

Liver and yeast ash added to diet equivalent to 5% of starting material; alfalfa ash = 20% dried alfalfa.

TABLE V.
Copper Absorption of Rats on Various Diets.

No. rats	Diet	Total Cu intake, μg/rat	Total Cu in feces, μg/rat
5	1718B	398	19
5	" + liver	875	68
11	Gray rats on various diets	860	54.6
13	Non-gray rats on same diets as above	912	99

The intake and fecal excretion of Cu are presented as the totals for a 3-day period.

Both the gray and non-gray rats were on several diets, some of which usually did, and some of which usually did not prevent graying.

copper. The addition of 5% liver or yeast to Diet 1718B increased the copper content to 20 and 18.3 μg per g respectively, amounts sufficient to account for their achromotrichia preventing effect according to the amount of copper necessary when added to the diet as $CuSO_4 \cdot 5H_2O$ (Table III).

Effect of ashing. The fact that most, if not all, of the achromotrichia preventing effect of liver, yeast and alfalfa was due to their mineral content was confirmed by dry ashing these materials and adding the ash to the 1718B diet in amounts equivalent to effective concentrations of unashed material (Table IV).

Intestinal absorption of copper. It is to be noted that the addition of quite small amounts of copper, 2.5 μg per g, to the 1718B diet produced a striking reduction in the incidence of achromotrichia. This was true whether the copper was added as copper sulfate or as liver or yeast. The fact that such small amounts of copper produced such large changes even though the 1718B diet already contained more than 10 times this amount of copper suggested that some of the copper in the 1718B diet might be in a form unavailable for absorption. This was checked by determining the dietary intake and fecal excretion of copper. The results (Table V) make it evident that intestinal absorption of copper was quite efficient whether the rats were on achromotrichia producing or achromotrichia preventing diets. Since the feces are the principal route of copper excretion (10), it is probable that these rats were retaining most of the dietary copper. That efficiency in intestinal absorption of copper was not a determining factor in the development of achromotrichia was supported further by a comparison of the last two groups of rats in Table V. These rats were selected from a number of experiments using various diets some of which usually did and some of which usually did not prevent achromotrichia. It is clear that there was no significant difference in the efficiency with which these two groups retained copper.

Hematocrits. Hematocrit determinations on 30 rats after 8 weeks on the 1718B diet gave an average value of 46.3. Ten comparable rats on the copper supplemented 1718B diet showed an average hematocrit of 45.0. Other gray rats were observed for as long as 5 months without showing evidence of anemia.

Treatment Experiments. Rats with gray hair showed no tendency to revert spontaneously toward their normal black hair color when maintained for long periods on diet 1718B. Even shaving, to stimulate new hair growth, produced only more gray hair. In many animals the fur coat did not completely regenerate leaving bald areas after shaving. Gray rats began to grow black hair quickly if their diet was changed to Purina Lab Chow or if the diet 1718B was supplemented with 5% yeast or liver, 20% alfalfa or 0.02% $CuSO_4 \cdot 5H_2O$. Black hair was first noted about 10 days after the diets were changed

10. Lindow, C. W., Peterson, W. H., and Steenbock, H., *J. Biol. Chem.*, 1929, v84, 419.

11. Elvehjem, C. A., *Physiol. Rev.*, 1935, v15, 471.

and became progressively more pronounced and more complete within periods of 3 months. Purina Lab Chow produced complete return to a normal hair coat within this period. The 1718B supplemented diets produced complete regeneration in some animals but in others their effect was definite but incomplete. Usually it was the rats with far advanced grayness which failed to show complete regeneration of black hair. Rats with less severe achromotrichia often returned to normal within one month.

Discussion. It is clear that copper was the principal factor in the achromotrichia observed in these experiments. Copper is known to be an essential dietary element(11) and is essential for melanin formation since dopa-oxidase is a copper protein complex(12). Likewise, several reports have indicated that black hair contains more copper than light colored hair(13) although a few reports to the contrary have appeared(14). However, several considerations suggest that a factor or factors in addition to simple copper deficiency may have exerted some influence in the achromotrichia observed in these experiments. Rats subsisting on the Purina Lab Chow ration were never observed to develop graying. On the other hand, rats on the purified diets containing equivalent amounts of copper occasionally did develop some graying. Likewise in treatment experiments none of the copper supplemented purified diets were as effective in regenerating black hair as was the Purina Lab Chow. Occasionally rats on the 1718B diet supplemented with 5% liver developed mild grayness which would not respond to additional copper. It should also be noted that liver, yeast, alfalfa and wheat germ produced considerable growth stimulation. This effect was still greater after 8 weeks than at the 4-week period recorded in Table II. Copper, the ash from liver, yeast and alfalfa, the known vitamins, additional protein or fat, different carbohydrates and additional minerals were all ineffective in duplicating this growth effect.

It is probable that copper is required for normal hair growth in addition to melanin production. Gray rats in these experiments never developed spontaneous alopecia but often showed diffuse thinning of the hair with an unkept, dull appearance. If the hair was shaved, deficient hair regrowth leading to partial alopecia was observed. Copper seemed to stimulate both melanin production and hair growth when added to the diet of gray rats.

The quantitative requirement of the rat for copper under the conditions of our experiment can be calculated from the data in Tables II and III plus data on the daily food intake which were recorded for many animals. In terms of concentration in the diet, it is evident that 14.6 μg of copper per g of a diet supplying approximately 4 calories per g was adequate for normal growth and hemoglobin production, although approximately 20 μg of copper per g of diet was required to prevent achromotrichia. Based on the number of grams of food consumed per day, rats weighing an average of 150 g maintained body weight and hemoglobin but became gray on a daily copper intake of 0.143 mg and maintained their black hair color if the copper intake was 0.196 mg per day. Gortner(2) also noted that more copper was required to prevent achromotrichia than to prevent anemia. According to his data, 0.02 to 0.12 mg of copper per day was required, this apparently being in addition to an unspecified amount in the diet. Kiel and Nelson(1) found that 0.05 mg of copper per day would cure achromotrichia in rats on an iron supplemented whole milk diet which supplied 0.24 mg of copper per liter. Assuming that a 150 g rat would consume 50 ml of milk per day, achromotrichia was cured with 0.06 mg per day in their experiments, an amount considerably less than that necessary in our experiments. This is contrary to the results of Drabkin and Waggoner(15)

12. Cunningham, I. J., *Biochem. J.*, 1931, v25, 1267.

13. Sarata, U., *Japan. J. Med. Sci. II Biochem.*, 1935, v3, 79; Yoshikawa, H., *ibid*, 1937, v3, 195, 269; Cohen, G. N., *Trav. membres soc. chim. biol.*, 1941, v23, 1504.

14. Sacchardi, P. and Giuliani, G., *Biochim. terap. sper.*, 1935, v22, 169.

15. Drabkin, D. L., and Waggoner, C. S., *J. Biol. Chem.*, 1930, v89, 51.

who reported that low copper synthetic rations were more effective than whole milk diets in curing anemia. Others have disputed this finding however(11).

Conclusions. Black rats subsisting on a purified diet containing 14.6 μg of copper per g grew well and maintained normal hemoglobin levels but developed marked achromotrichia. The achromotrichia could be cured by adding copper sulfate to the diet and could be prevented by diets containing approximately 20 μg of copper per g. The activity of liver, yeast and alfalfa in preventing and curing the achromotrichia was due to their copper content. Evidence suggesting that the basal purified diet used in these experiments may have been deficient in an unidentified growth factor has been discussed.

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Formation of Neutralizing Antibody in Monkeys Injected with Poliomyelitis Virus and Adjuvants.* (17964)

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It has been long known that rhesus monkeys receiving repeated injections of poliomyelitis virus produce neutralizing antibodies. Recently Morgan, Howe, and Bodian(1) and Morgan(2) studied the effect of dosage, route of administration of the antigen and other factors on the hyperimmunization against this virus. No evidence was found that antibody formation was augmented by the incorporation of the Lansing type of virus into a water-in-oil emulsion with or without tubercle bacilli (1,3).

Our hope for the effectiveness of these adjuvants in relation to poliomyelitis virus was raised by results obtained recently with another neurotropic virus, namely, that of rabies(4). It was found that rabbits or guinea pigs, given a single injection of brain containing inactivated rabies virus emulsified in paraffin oil and one month later a few more injections of the same antigen in salt solution, produced neutralizing and complement fixing antibodies in great abundance.

Material and methods. As an immunizing agent, monkey spinal cord, containing active poliomyelitis virus of the Lansing type, was used. It was suspended in salt solution with the aid of a Waring blendor. The antigen was injected either in this form or emulsified in paraffin oil with or without the addition of a small amount of killed *Myco. butyricum*. The technic of emulsification and the ingredients used have been described previously (5). Immature rhesus monkeys (*Macacca mulatta)* as a rule, were given 5 simultaneous injections. The sites were the pectoralis, and the flexor muscles of the femur and the subcutaneous tissue of the nuchal region. One monkey (Group IIIa) received the primary injection in the footpads. The "booster" injections were the same for all groups, namely, the antigen in salt solution. The data are given in Table I.

The neutralizing antibodies were titrated using 4 or 5 fold serial dilutions of serum

* Aided in part by a grant from The National Foundation for Infantile Paralysis, Inc.

1. Morgan, I. M., Howe, H. A., and Bodian, D., *Am. J. Hyg.,* 1947, v45, 379.

2. Morgan, I. M., *Am. J. Hyg.,* 1948, v48, 394; Morgan, I. M., *J. Immunol.,* 1949, v62, 301.

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4. Freund, J., Lipton, M. M., and Pisani, T. M., PROC. SOC. EXP. BIOL. AND MED., 1948, v68, 609; Lipton, M. M., and Freund, J., *J. Immunol.,* 1950, v64, 297.

5. Freund, J., Thomson, K. J., Hough, H. B., Sommer, H. E., and Pisani, T. M., *J. Immunol.,* 1948, v60, 383.

TABLE I.
Material Injected per Monkey. (First injection).

Group	No. of monkeys	Lansing virus monkey cord, g	Salt sol., ml	Paraffin oil, ml	Arlacel A, ml	*Myco. butyricum*, mg
I	6	1	9	0	0	0
II	6	1	4	4.5	.5	.1
III	6	1	4	4.5	.5	0
IIIa	1	0.4	1.6	1.8	.2	0
		Booster injections 5 and 11 wk after primary injection				
All monkeys		1	10	0	0	0

TABLE II.
Neutralizing Antibody Titers in Monkeys Injected with Poliomyelitis Virus With and Without Adjuvants. Dilution of sera before adding equal volumes of virus dilution.

Group	Immunizing antigen: Lansing virus with	Rhesus No.	Neutralizing antibody (serum endpoint)* 1 mo. after 1st inj.	Mean	2 wk after 2nd inj.†	Mean	1 wk after 3rd inj.†	Mean
I	Saline	1	2.4, 1.9	1.9	3.4, 3.6	3.1	3.6	3.4
		2	2.4, 2.5		3.3, 3.3		2.9	
		3	2.1		3.7, 3.4, 3.4		3.7	
		4	1.7		3.3		3.7	
		5	1.7		2.6		3.1	
		6	1.3, 1.8		2.6		3.3	
II§	Paraffin oil	7	3.8	3.3	4.4, 4.3	4.2	4.6, 4.3	4.3
	Arlacel	8	3.7		4.4, 4.3, 4.0‡		—	
	Myco. butyricum	9	3.3		4.3, 4.5		4.2	
		10	2.7, 2.5		3.8‡		—	
III	Paraffin oil	11	3.3	3.1	3.9	3.9	3.6, 3.5	3.7
	Arlacel	12	3.3		3.9, 3.5		3.6	
		13	2.9		4.1, 4.1		3.8	
		14	2.9		3.8		3.7	
		15	2.8		3.9		3.9, 3.6	
		16‖	3.3, 3.3		4.0		3.6	

* = Reciprocal log of the highest dilution of serum neutralizing the virus, 50% of mice being protected. (Reed and Muench formula).
† = 2nd and 3rd inj.; virus in saline (lg. Rh. CNS).
‡ = Titers 1 wk after 2nd inj.
§ = Two animals died of allergic encephalitis before the 1st bleeding.
‖ = Group IIIa 1st inj.; 4 ml in footpads.

mixed with a constant amount of virus; the mixture was held at room temperature for 1 hour and injected into the brain of mice in groups of 8 or 9. Although the virus suspension used in all tests was from the same pool, the number of LD_{50} doses of virus ranged from 10 to 100 (calculated from the final dilution of virus *after* the latter was added to serum). The mice were observed for 4 weeks. All deaths occurring after the first day were considered as specific deaths. The serum 50% endpoints were calculated by the method of Reed and Muench(6). The titers are expressed as the serum dilution endpoints *before* the addition of an equal volume of virus dilution.

Results. The mean antibody titers for each group of monkeys are given in Table II. One month after the first injection they were about 10 times higher in the 2 groups receiving adjuvants than in the group given the saline suspension. Two weeks after the second injection, the titers rose in all groups, but a conspicuous difference was still sustained. One week after the third injection, the mean levels were only slightly different

6. Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, v27, 493.

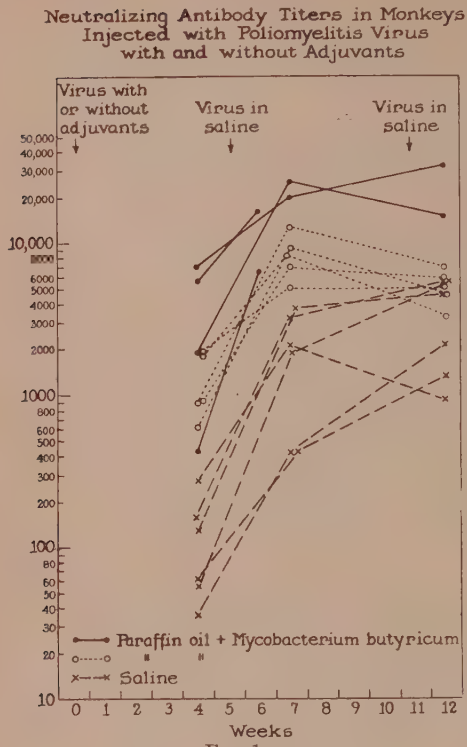

FIG. 1.

from those found in the preceding test. However, since the mean titers rose slightly in the group with saline suspension and fell slightly in the group with oil in the absence of *Myco. butyricum*, the difference between these 2 groups became less.†

In Table II, the multiple figures under the same heading represent titers found on repeating the tests. Some of the samples were titrated in the two different laboratories by different persons.

Fig. 1 presents graphically the data of Table II using the average values for samples titrated repeatedly.

As customary, with each neutralization test, the infectivity of the virus suspension was titrated. Groups of 8 or 9 mice were injected with 10-fold serial dilutions of the virus suspension beginning with 10^{-2} and including 10^{-5} dilutions. The arithmetic mean titer was $10^{-3.5}$ (range $10^{-3.1}$ to $10^{-4.0}$). Accordingly, as mentioned above, the LD_{50} values in the different tests varied from 10 to 100. Since some of the samples of the sera were titrated repeatedly, a tabulation of the results may reveal the effect of the variation in the concentration of virus on neutralizing titers obtained. Table III shows that within this range, this variation had little if any effect on the serum titers.

In another experiment, using 4 monkeys, the synergetic effect of paraffin oil was confirmed. Table IV includes a monkey (No. 20) which had an inapparent infection following intracerebral injection of Lansing virus, as shown by the presence of antibody, before immunization was started. The titer of the serum before immunization was log 2.5 and reached the unusual titer of log 4.6 4 weeks after the first injection.

In 4 of the 6 monkeys which were given *Myco. butyricum* in addition to infected spinal cord and paraffin oil, fatal allergic encephalitis occurred. Two of them died before the first bleeding. One monkey had some of the symptoms of this experimental malady, while in another one no symptoms were seen. This complication was not unexpected in view of the experience of Morgan(3) and Kabat, Wolf, and Bezer(7) with tubercle

TABLE III.
Antibody Titer and Concentration of Virus in Test

Serum	Log. of LD_{50} doses of virus					
	1.0	1.3	1.4	1.5	1.7	2.0
6–I*				1.8		1.3
1–I		2.4			1.9	
12–II	3.9		3.5			
8–II	4.4		4.0	4.3		
15–III			3.9		3.6	
7–III					4.6, 4.3	
1–II	3.6			3.4		
10–I		2.7			2.5	
7–II	4.4				4.3	
2–II	3.3			3.3		
13–II	4.1			4.1		
16–I				3.3		3.3
3–II	3.4			3.7	3.4	
2–I		2.4			2.5	
11–III			3.5			3.6
9–II	4.3			4.5		

* 6–I = Rhesus No. 6, 1st bleeding.

† One week after the third immunizing injection, all the monkeys were challenged by intracerebral injection of 3000 LD_{50} doses of Lansing virus. None developed signs of poliomyelitis.

TABLE IV.
Neutralizing Antibody Titers in Monkeys Injected with Poliomyelitis Virus Combined with Paraffin Oil and Arlacel A. Dilution of sera before adding equal volumes of virus dilution.

Rhesus No.	Before immunizing inj.	4 wk after 1st inj.	8 wk after 2nd inj.
17	0†	4.1	3.6
18	0	3.3	3.9
19	0	2.4	3.9
20*	2.5	4.6	3.9

* Inapparent infection following intracerebral inoculation.
† Undiluted serum did not neutralize the virus.

bacilli and our own in guinea pigs using either species of acid-fast bacilli(8). In the present experiment, the *Myco. butyricum* was used since it may be a slightly more effective potentiating agent(5,9) than tubercle bacillus. A relatively small amount was injected, 0.1 mg per monkey with the hope of diminishing its untoward effect.

In the present experiments, the "booster" injections were of antigen in saline. With rabies virus high antibody titers were obtained by one injection of antigen in water-in-oil emulsion followed by a "booster" injection of antigen in saline(4). Following an injection of a bacterial antigen in water-in-oil emulsion, the injection of antigen in saline was as effective a "booster" as antigen emulsified in paraffin oil(5).

Conclusions. 1. The formation of neutralizing antibodies in rhesus monkeys is enhanced when the antigen, spinal cord containing poliomyelitis virus, is incorporated into a water-in-oil emulsion. 2. The addition of a small amount of killed *Myco. butyricum* to the water-in-oil emulsion further augments antibody formation. Some of the monkeys, however, develop fatal allergic encephalitis. 3. By repeating the injections of antigen without these adjuvants, antibody titers can be produced which are equal to those found after the injection of antigen in water-in-oil emulsion, but not as high as in monkeys receiving the combination of antigen, paraffin oil and killed *Myco. butyricum.*

The technical assistance of Joyce Paulnock and Hugh Wiles is gratefully acknowledged.

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Lack of Effect of Lactogenic Hormone on Mammary Adenocarcinoma in Mice.* (17965)

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Many clinical observations have indicated that the growth of mammary cancer in women is accelerated by pregnancy and lactation (1,2). The effect of lactation alone is not known, although Geschickter(3) believes that it does not influence the growth rate of infiltrating cancer of the breast. Pregnancy stimulation is usually ascribed to the high estrogen titre, a situation which does not apply during lactation(3).

In mice, estrogens exert a carcinogenic effect on the mammary gland(4), but the estrogen preponderance of pregnancy ap-

* Aided by grants from the Research Board of the University of California.

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2. Harrington, S. W., *Ann. Surg.,* 1937, v106, 690.
3. Geschickter, C. F., Diseases of the Breast, 2nd ed., 1947, J. B. Lippincott, Philadelphia, 483-486.
4. Lacassagne, A., *Compt. Rend. Acad. de Sci.,* 1932, v195, 630.

TABLE I.
Effect of Pure Pituitary Lactogenic Hormone and Castration on Mammary Adenocarcinoma.
Dissected tumor wt in g.

Saline-injected		Hormone-treated	
Intact	Castrate	Intact	Castrate
.16	1.45	2.47	.27
1.52	.15	1.10	.48
.66	.24	2.45	1.22
1.19	2.11	.41	.05
1.26	.03	.40	1.71
1.82	2.86	.03	1.25
1.82		.16	.49
.02		.31	
1.06 ± 0.24*	1.14 ± 0.48	0.92 ± 0.35	0.78 ± 0.22

*Mean value ± standard error.

parently does not accelerate the tumor growth(5). The influence of lactation, with its high lactogenic titre, has not been established, although it has been shown that a crude prolactin preparation did not modify the growth rate of these tumors(6). With the recent availability of homogeneous pituitary lactogenic hormone, it was thought worth while to test this substance for its possible effect upon the growth of mammary adenocarcinoma in mice.

Procedure. Virgin female mice of the C3H strain, 5 to 6 months old, were used. To obviate any ovarian influence half the group was oophorectomized. After a 7 week recovery period, uniform fragments of a transplantable C3H strain mammary adenocarcinoma were implanted by trocar in the dorsal subcutaneous tissues. Eight days later subcutaneous injections were begun. Half of each group, castrated and intact, received two 0.1 cc injections daily, each containing 1 mg of purified lactogenic hormone assaying 30 I.U. per mg(7). The control groups received equal volumes of normal saline. Periodic recordings were made of body weights and tumor diameters. Daily vaginal smears of the hormone-treated mice showed anestrous periods of 12 to 15 days occurred regularly throughout the experiment, thus demonstrating the continued potency of the hormone. After 41 days of injection the experiment was terminated, and the tumors were dissected free and weighed. Necropsy revealed no gross visceral metastases. Of the original 40 mice in the experiment, eight were disqualified for developing spontaneous tumors, and three died of intercurrent disease.

Results. The weights of the dissected tumors are shown in Table I. All tumors had grown considerably since transplantation, although there was great variation between those of individual animals. The tumors of the hormone-treated animals were slightly smaller than those of the controls, but the difference is not statistically significant.

Since neither the hormone nor castration significantly altered the final tumor weight it is interesting to combine the various groups into the categories shown in Table II and note the similarity of results with these larger groups. The hormone did not affect body weight gain.

Conclusion. Homogeneous pituitary lactogenic hormone (2 mg injected daily for 41 days) did not significantly modify the growth rate of a transplanted mammary adenocarcinoma in C3H mice. Castration after sexual maturity did not alter the growth rate of these tumors.

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TABLE II.
Combinations of Original Groups to Show Effect of Lactogenic Hormone and Castration on Tumor Growth and Body Weight Gain. Mean values in g.

Group	No. in groups	Body-wt increment	Tumor wt
All saline-treated (castrated and intact)	14	2.6	1.09
All hormone-treated (castrated and intact)	15	2.5	.85
All intacts (control and hormone-treated)	16	3.4	.98
All castrates (control and hormone-treated)	15	1.3	.95

Incomplete Growth Cycle of Influenza Virus in Mouse Brain. (17966)

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Stuart-Harris(1) and Francis and Moore (2) were able to propagate the WS strain of type A influenza virus in mouse brain. Once adapted to this organ, the virus multiplied and produced fatal encephalitis after intracerebral inoculation. On the other hand, repeated attempts to propagate other strains of influenza virus in mouse brain have failed (2,3,4). Yet, despite the absence of readily demonstrable neurotropic tendencies, active preparations of several egg-adapted strains have been found to have two striking effects after intracerebral inoculation in mice: (a) "toxicity"(3), and (b) the ability to interfere with certain unrelated neurotropic viruses, *e.g.*, equine encephalomyelitis viruses(4). Both effects, especially "toxicity," depend on the inoculation of relatively large amounts of virus.

It was thought that a possible explanation of the interference between influenza and unrelated viruses might be found in the form of common cellular receptors. In studies on the effect of infection with influenza viruses on the amount of virus hemagglutination inhibitor (VHI) extractable from a variety of tissues, no relationship between VHI and equine encephalomyelitis virus could be detected(5). It was found, however, that mouse brain was a particularly rich source of VHI. Progressive destruction of VHI was noted after intracerebral inoculation not only of the neurotropic WS variant (WS–N) but also, to a lesser degree, of the non-neurotropic WS, PR8, or Lee strains(5). It appeared difficult to reconcile this observation with the fact that the latter strains did not multiply, but, on the contrary, progressively decreased in titer in the mouse brain. The measurable decrease in VHI, which was assumed to be due to progressive destruction of cellular receptors of the brain, suggested some viral activity which could not be accounted for by the amount of infective virus present.

Reports to the effect that after inoculation of influenza virus into the allantoic cavity of chick embryos, the appearance of newly formed, infectious virus was preceded by that of specific complement-fixing antigen and viral hemagglutinin(6,7) stimulated the idea that influenza virus might undergo in the mouse brain an incomplete cycle of reproduction in which the development of virus particles did not proceed beyond the hemagglutinating to the infectious stage. This hypothesis has been borne out by experimental findings.

Materials and methods. Virus strains: The PR8 and WS strains of type A, and the Lee strain of type B influenza virus were propagated in the allantoic cavity of 11-day chick embryos. Eggs inoculated with infected allantoic fluid, diluted 10^{-6}, were incubated for 2 days at 36°C, then chilled overnight at 4°C before harvest. Freshly harvested virus was used, which was sometimes concentrated by centrifugation for 1 hour at 13,000 r.p.m. in the cold and resuspension of the sediment in a reduced volume of the supernatant. The neurotropic variant of the WS strain (WS–N) was originally obtained from Dr. T. Francis, Jr., and has been maintained by intracerebral passage in mice. Stock virus suspensions consisted of 20% infected mouse brain in 50% heated normal rabbit serum in saline.

Cholera vibrio filtrates: Culture 35A3 of

1. Stuart-Harris, C. H., *Lancet*, 1939, v1, 497.
2. Francis, T., Jr., and Moore, A. E., *J. Exp. Med.*, 1940, v72, 717.
3. Henle, G., and Henle, W., *J. Exp. Med.*, 1946, v84, 623.
4. Vilches, A., and Hirst, G. K., *J. Immunol.*, 1947, v57, 125.
5. Schlesinger, R. W., to be published, see Abstracts of papers, *Soc. Am. Bact.*, 49th Gen. Meeting, 1949, p. 94.
6. Hoyle, L., *Brit. J. Exp. Path.*, 1948, v29, 390.
7. Henle, W., and Henle, G., *J. Exp. Med.*, 1949, v90, 23.

V. cholerae (Inaba) was obtained from the National Institute of Health, Bethesda, Md. Berkefeld N filtrates of 18 hour beef infusion broth cultures were used as source of "receptor-destroying enzyme" (RDE)(8). Such preparations were capable of destroying, in 1 hour at 37°C, all hemagglutinin inhibitors demonstrable in mouse brain extracts(5).

Infectivity titrations: Ten-fold serial dilutions of test materials were inoculated into 11- or 12-day-old eggs. Fluids were harvested after 48 hours' incubation at 36°C and tested for hemagglutinin (HA). Infectivity titers were estimated on the basis of HA production and will be expressed as EID_{50} per gram of tissue or per ml of fluid(9). Intracerebral titers of the WS–N strain are also given in terms of LD_{50} per gram of infected brain tissue.

Hemagglutinin titrations: To 0.5 ml of each 2-fold serial dilution of test material, 0.5 ml of a 0.4% (sometimes 0.3%) suspension of fowl erythrocytes (RBC) was added. All dilutions were made in saline or, when the test material contained RDE, in saline containing 1 or 2% sodium citrate(10). The tests were read as soon as the cells in control tubes had settled in a clearly demarcated button, usually after 1 hour(11). Agglutination was read as either complete or partial, with partial agglutination usually limited to one tube. The endpoint was either the highest dilution giving complete HA or the logarithmic mean between the highest dilution giving partial HA and the next lower dilution. Titers will be expressed as the inverse log of the final concentration (after addition of cells) of test material.

Partial purification by adsorption and elution. Because the presence of tissue components tended to obscure the sedimentation pattern in HA titrations, it was desirable to eliminate them. Fowl RBC to a final concentration of 1.5% were added to the test material. If the latter contained RDE, sodium citrate to a final concentration of 1% was added. The mixture was kept at 4°C for 1 hour to allow adsorption, then the cells were sedimented and resuspended in the desired volume of RDE. Incubation in the 37°C waterbath for 60 to 90 minutes resulted in elution of all adsorbed HA.

Complement-fixation tests: Crude 1:4 mouse brain suspensions in saline were centrifuged at 13,000 r.p.m. for 1 hour, and the clear supernatant was used as C-F antigen. Presumably, these preparations contained mainly the "soluble" antigen(12). 0.1 ml of each 2-fold antigen dilution and 0.1 ml of a constant dilution of type-specific or of control human serum were mixed with 0.2 ml of complement dilution representing 1.5 to 2 units. After incubation for 1 hour at 37°C, 0.2 ml of a 1.5% sensitized sheep erythrocyte suspension was added. The tests were read after further incubation for 30 minutes at 37°C. The highest dilution of antigen giving partial fixation was chosen as endpoint. If the highest positive dilution gave complete (4+) fixation, the endpoint was considered as 0.15 log higher. Antigen titers will be expressed in terms of final concentration of brain tissue before addition of the hemolytic system.

Experimental. "Unmasking" of virus hemagglutinins after digestion of brain inhibitors by cholera vibrio filtrates: Previous experiments had revealed VHI of high titer in mouse brain extracts(5). While infection with the neurotropic strain, WS–N, reduced the titer progressively to about 5% of the normal value by the 3rd to 4th day after inoculation, there remained enough VHI in the tissue to inhibit any demonstrable hemagglutinin in brains which contained 10^8 LD_{50} or more of the virus. Since it had been found (5) that brain VHI, like red cell receptors and inhibitors from other sources, could be destroyed by the receptor-destroying enzyme (RDE) in cholera vibrio filtrates(8), the effectiveness of RDE in "unmasking" hemag-

8. Burnet, F. M., McCrea, J. F., and Stone, J. D., *Brit. J. Exp. Path.*, 1946, v27, 228.

9. Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, v27, 493.

10. Stone, J. D., *Austr. J. Exp. Biol. and Med. Sc.*, 1947, v25, 137.

11. Salk, J. E., *J. Immunol.*, 1944, v49, 87.

12. Wiener, M., Henle, W., and Henle, G., *J. Exp. Med.*, 1946, v83, 259.

TABLE I.
"Unmasking" of PR8 Virus Hemagglutinin from a Mixture of Virus and Mouse Brain by RDE.

Test material	Medium	Log HA titer* after overnight storage at 4°C	Log HA titer* after overnight storage at 37°C with RDE	Log, EID/ml*	EID/HA ratio (log)
PR8 AF 1:50	Saline	3.9	3.9	10.2	6.3
" " "	25% NMB	<2.0	3.8	10.2	6.4

* Titers are expressed as inverse log of final dilution of original allantoic fluid concentrate (centrifuged 1 hr at 13,000 rpm, sediment resuspended in 1/10 vol. supernate).

TABLE II.
Infective, Hemagglutinating, and Complement-fixation Antigen Titers of Mouse Brains Harvested 3 Days After Intracerebral Inoculation of the Neurotropic WS strain.

Intracerebral inoculum LD_{50}/0.03 ml (log)	Titers* (log) of brain tissue				
	LD_{50}/g	EID/g	HA	C-F antigen	Ratio EID/HA
6.0	7.2	8.5	2.3	1.5	6.2
5.0	8.0	8.0	2.3	1.65	5.7
3.0	8.5	8.0	2.1	1.5	5.9
1.0	7.7	8.2	1.5	0.9	6.7

* Titers are expressed as inverse log of the final concentration of brain tissue (by wet wt).

glutinins in mixtures of virus and mouse brain suspensions was tested. To a 1:4 suspension of homogenized normal brain tissue in saline was added 1/50 volume of PR8-infected allantoic fluid. A portion of the mixture was kept in the refrigerator. To the remainder, equal parts of RDE were added, and the mixture was kept in the 37°C waterbath overnight. Similarly, virus diluted 1:50 in saline was incubated with RDE at 37°C overnight. The next day, all samples were tested for hemagglutinin (HA).

Table I shows that RDE under these conditions failed to affect the HA titer of the virus diluted in saline. However, while no HA was detectable in the untreated brain-virus mixture, overnight incubation in presence of RDE "unmasked" HA to full titer. Further tests revealed that incubation of brain-virus mixtures with RDE for 1 hour was sufficient to obtain 100% recovery of known amounts of virus HA, even without addition of Ca ions(13).

Infective, HA, and C-F antigen titers in mouse brains after intracerebral inoculation of the WS–N strain. Treatment of brain suspensions with RDE was used in several experiments with the WS–N strain in an attempt to determine whether HA could be demonstrated also in tissue infected *in vivo*.

In a typical experiment, mice were inoculated intracerebrally with varying amounts, ranging from 10^1 to 10^6 LD_{50}, of WS–N stock virus. On the third day, 5 to 7 mice of each group were sacrificed. Their brains were pooled and 25% suspensions were prepared. Portions of each suspension were used for the titrations summarized in Table II. All of the groups yielded titers of the same order of magnitude in each type of test, indicating that the yield at the peak of virus multiplication was not affected by the size of the intracerebral seed inoculum. Of particular interest, in the light of the data to be described below, is the fact that the EID/HA ratio was about 10^6 in each instance. Thus, after digestion of infected brain tissue with RDE, it was possible to show that this strain of virus has retained the EID/HA ratio characteristic of the WS and other strains of influenza virus grown under optimal conditions in the allantoic cavity of chick embryos.

Infective, HA, and C-F antigen titers in mouse brain after intracerebral inoculation of non-neurotropic strains of influenza virus.

13. Burnet, F. M., and Stone, J. D., *Austr. J. Exp. Biol. and Med. Sc.*, 1947, v25, 227.

TABLE III.
Infective, Hemagglutinating, and Complement-fixation Antigen Titers of Mouse Brains Harvested at Varying Intervals After Intracerebral Inoculation of PR8-infected Allantoic Fluid.

	Titers (log)				
		Hemagglutinin after overnight at			
Test material	EID/g	4°C	37°C with RDE	C-F antigen	Ratio EID/HA
Seed virus (all. fluid)	10.2	3.9	3.9	n.t.†	6.3
M.B. 1 hr*	7.2	<.9	<1.8	<0.9	>5.4
" 5-6 hr	7.1	<.9	1.95	1.05	5.15
" 24 "	6.0	<.9	3.0	1.5	3.0
" 48 "	5.1, 5.3‡	<.9	3.45	1.8, 1.5‡	1.75

For explanations see Table II.
* M.B. = Mouse brain.
† n.t. = not tested.
‡ Duplicate figures indicate tests on two separate brain pools.

Entirely different results were obtained with brains of mice inoculated intracerebrally with the non-neurotropic WS, PR8, or Lee strains. As had been previously found by others(3,4), only a fraction of the inoculum could be recovered as infectious virus from brains harvested within 1 or 2 hours after inoculation. This fraction amounted to about 1 to 10% of the amount theoretically recoverable if one assumed that all of the inoculum remained unaltered within the brain.* This initial drop in recoverable infectious virus was followed by further progressive decrease in titer during subsequent days (Table III). The infective titer of brain suspensions was not increased by incubation with RDE. In contrast, as shown in Table III, treatment with RDE revealed the presence of HA in samples in which none was demonstrable without treatment. Since the seed virus had an HA titer of $10^{-3.9}$, the maximum theoretical yield at 1 hour (without virus multiplication) would have been $10^{-2.8}$. The actual titer was less than $10^{-1.8}$ (lowest dilution tested). However, HA was demonstrable after 5 to 6 hours. Two days after inoculation the titer was 4-fold higher than the theoretical maximum and at least 45-fold higher than at 1 hour. The significance of this increase is underlined by the shift in the EID/HA ratio from $10^{6.3}$ for the allantoic fluid inoculated to $10^{1.75}$ for the brains harvested 2 days after inoculation. Simultaneously with the increase in HA, specific complement-fixing antigen became demonstrable in the mouse brains.

Production of viral hemagglutinin in mouse brain in a single cycle of multiplication. Fig. 1 shows that after intracerebral inoculation of PR8 virus, hemagglutinin was not demonstrable at 1 to 3 hours but began to appear after 4 hours. It increased rapidly up to 9 hours, after which there was no appreciable further rise. The maximum titer was about 100-fold higher than the initial amount. Similarly, after intracerebral inoculation of the PR8 or WS strains, any significant increase in C-F antigen was completed within about 8 hours after inoculation. In contrast to the findings for the type A strains, it was found that the constant period preceding any demonstrable

* One mouse brain weighs approximately 0.4 g and has a volume of about .4 ml *in situ*. Inoculation of .03 ml into this organ would therefore result in 13-fold dilution of the inoculum. Hence, the maximum amount theoretically recoverable before multiplication begins would be 1.1 log less than the inoculum. Initial losses of 90 to 99% of the theoretically recoverable virus have been described for equine encephalomyelitis virus in mouse brain (14) and also for influenza viruses in the chorioallantoic membrane of the chick embryo(15). Since in the case of the mouse brain it is impossible to control the amount of leakage or to separate adsorbed from free virus, nothing can be surmised concerning the nature of this initial loss.

14. Schlesinger, R. W., *J. Exp. Med.*, 1949, v89, 491.

15. Henle, W., *J. Exp. Med.*, 1949, v90, 1.

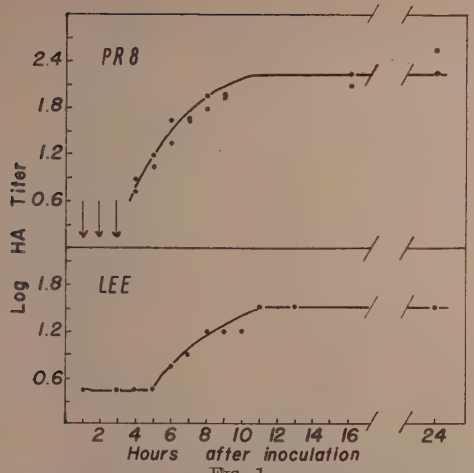

FIG. 1.

Rise in HA titer in mouse brain after intracerebral inoculation of allantoic fluids infected with PR8 or Lee virus.

increase was at least 6 hours when the Lee strain was used for intracerebral inoculation. Fig. 1 shows that the highest titer was reached after 11 hours and was maintained up to at least 24 hours after inoculation.

These results, especially the characteristic difference between the PR8 and Lee strains, led to the tentative conclusion that the HA and the C-F antigen were products of a process analogous to a single growth cycle in the allantoic membrane(16) which, in the mouse brain, did not go on to the completion of infectious virus particles. On passage to other mice, the HA failed to further reduplicate itself, just as it failed to infect chick embryos. In this respect, the material derived from mouse brains appeared to be similar to the hemagglutinating, but non-infectious "500 S component" present in allantoic fluid of eggs inoculated with PR8 virus in high concentration(17). However, while the allantoic membrane is known to support the propagation of fully infectious virus, especially after inoculation of more dilute seed virus(18), this was not true for non-neurotropic strains in mouse brain. In fact, if the cells involved in this incomplete cycle of multiplication were inherently unable to support the development of *complete* virus particles, then the inoculation of graded amounts of virus should result in proportional differences in yield of hemagglutinin and C-F antigen. This expectation was fulfilled in several tests with the WS and PR8 strains. An example is illustrated in Table IV. In this experiment, the inocula differed by a factor of 4, and so did the yields at 48 hours after inoculation. Only in the range of low seed dilutions were the yields nearly identical (see the first two lines in Table IV). Presumably in this range a maximum number of cells is saturated with virus. An attempt to estimate the actual increase of HA units in mouse brain is defeated by the uncertainty concerning the number of seed particles involved in infection. Thus, while after inoculation of various dilutions of PR8 virus the increase over the theoretical maximum yield was only 13-fold (Table IV), the increase over the amount present at 1 to 3 hours after inoculation of the same undiluted allantoic fluid was about 100-fold (Fig. 1). In other experiments, with the PR8 or WS strain, increases of up to 38-fold over the theoretical maximum were observed. Since this figure is based on the highly unlikely assumption* that each infectious unit injected is capable of initiating multiplication, the increase may be assumed to be much higher and well in the range of the yields given by Henle *et al.*(16) for "one-step" growth experiments in eggs. Variations in maximum yield reflecting variations in the intracerebral seed inoculum were found also for C-F antigen. However, because of its low titer under the most favorable conditions, the range of seed dilutions giving rise to *any* demonstrable C-F antigen in mouse brain was too narrow to be conclusive by itself.

Properties of the "incomplete" virus. The hemagglutinin recovered from mouse brain is adsorbed onto and agglutinates fowl and human type O erythrocytes. It elutes spontaneously and, as already mentioned, is removed from red cells rapidly and quantitatively by RDE. It appears to be antigenic in

16. Henle, W., Henle, G., and Rosenberg, E. B., *J. Exp. Med.*, 1947, v86, 423.

17. Gard, S., and von Magnus, P., *Ark. Kemi, Miner. och Geol.*, 1947, v24b, No. 8.

18. Henle, W., and Henle, G., *Am. J. Med. Sc.*, 1944, v207, 705.

TABLE IV.
Effect of Varying the Amount of PR8 Virus Inoculated Intracerebrally on the Yield of Hemagglutinin in Mouse Brain 48 Hours After Inoculation.

Intracerebral inoculum—PR8 allantoic fluid		Yield in M.B. at 48 hr (log)		
Dilution	Log HA titer of inoculum	Theoretical max.*	Actual	Min. increase†
Undil.	3.15	2.05	2.55	0.5‡
1:4	2.55	1.45	2.55	1.1
1:16	1.95	.85	1.95	1.1
1:64	1.35	.25	1.35	1.1
1:256	.75	.00	.75	>0.75
1:1024	– .15	.00	<0.3	—

For explanations see Table II.
* See footnote 1.
† Min. increase = Difference between actual yield and theoretical max.
‡ Rate of hemagglutinin production after inoculation of the same undiluted allantoic fluid was shown in Fig. 1, which showed that the actual increase was at least 100-fold over the amount demonstrable up to 3 hr after inoculation.

that intravenous inoculation into a rabbit of 10 ml of WS mouse brain HA, partially purified by adsorption-elution and centrifugation, resulted in production of specific HA-inhibiting antibody. The preparation used as antigen contained a small proportion of residual infectious WS virus, presumably not enough to account for the antigenicity(19). Further work on the antigenic specificity of mouse brain hemagglutinins is in progress.

Preliminary attempts have been made to differentiate the HA in mouse brain from fully developed virus, as it occurs in allantoic fluid, by means of centrifugation in a sucrose gradient according to the method described by Pickels(20). This method has failed to reveal a significant difference in sedimentation pattern, indicating that there is no gross difference in size. The similarity in size between the incomplete and the fully developed virus adds further weight to the conclusion that the HA present in mouse brain is not a degradation product of the inoculum but a product of a "stunted" growth process.

Further evidence in this direction has been obtained in recent experiments in which PR8-infected allantoic fluids of identical HA titers but differing infectious titers, obtained by the method of von Magnus(21) (serial passage in eggs with undiluted allantoic fluids), were inoculated into mice. The production of HA in the brains of these mice depended upon the *infectious* titer of the inoculum, not on the HA titer. Since the total amount of sedimentable material in such preparations parallels the HA rather than the infectious titer (17), it would appear that only particles capable of infecting cells and of self-duplication are capable of inducing the incomplete growth cycle in mouse brain. Details of this experiment will be reported in a subsequent paper.

It may be assumed that the C-F antigen from infected mouse brain is of the "soluble" type. The antigens were prepared without treatment with RDE and under conditions which eliminated most of the detectable hemagglutinin by centrifugation. No adequate complement-fixation tests have as yet been done with the sedimentable material.

Discussion. Mouse brain tissue has hitherto been considered as insusceptible to infection with viruses of the influenza group, with the exception of the neurotropic variant of the WS strain(1,2). The findings reported in this paper necessitate a partial revision of this belief. While mouse brain does not support the complete reduplication of unadapted strains, it nevertheless enables them to develop through certain phases of their repro-

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20. Hughes, T. P., Pickels, E. G., and Horsfall, F. L., Jr., *J. Exp. Med.*, 1938, v67, 941.

21. von Magnus, P., *Ark. Kemi, Miner. och Geol.*, 1947, v24b, No. 7.

ductive cycle which have been demonstrated to precede the completion of infectious particles in the allantoic endothelium of the chick embryo(6,7). After intracerebral inoculation of non-neurotropic strains, HA and C-F antigen appear and increase in spite of progressive decrease in infectious titer. The conclusion that this phenomenon results from a reproductive process rather than from viral degradation is based on the following considerations:

(a) The constant period preceding increase, and the rate and duration of the rise in hemagglutinin titer in mouse brain correspond for each strain to those characteristic for it in one-step growth experiments in eggs(16);

(b) the HA and C-F antigen titers remain for at least 48 hours at the levels reached at the end of the single rise period, without evidence of qualitative change, while the infectious titer continues to decrease;

(c) the yield of HA and C-F antigen in mouse brain varies in proportion to the amount of seed virus, as has been found also for the yield of infectious virus in one-step growth experiments in eggs(16,7);

(d) only fully infectious particles, but not the "500S" component(17), present in certain infected allantoic fluids, give rise to incomplete virus;

(e) the HA from mouse brain is of similar or of the same size as the complete virus in allantoic fluid.

It has already been mentioned that a valid estimate of the number of incomplete particles arising from each infecting particle cannot be made. The data reported leave no doubt, however, that the increases in HA titer are significant and may well be within the range of the yields estimated by Henle *et al.*(16) for single growth steps in eggs.

What are the factors responsible for this stunted reproduction? The work on the neurotropic variant of the WS strain shows that the process of adaptation has enabled it to utilize the cells of the mouse brain in much the same way as egg-adapted strains use the allantoic endothelium. More detailed comparison between the egg-adapted WS strain and its neurotropic analogue may give a clue concerning the nature of this adaptive process. At this stage, only a general working hypothesis can be developed, *i.e.*, that the infected cells lack some specific component or components which "fully susceptible" cells of other tissues contribute to the incomplete virus particles in order to make them infectious.

The demonstration of incomplete virus reproduction in mouse brain may throw new light on the mechanism of the "toxicity" of non-neurotropic influenza strains after intracerebral inoculation(3). It is likely that the integrity of infected cells is equally impaired whether the endproduct of virus multiplication is complete or "stunted" virus. Thus, even in the absence of multiplication in the classical sense, *i.e.*, increase in *infectious* titer, the "toxicity" appears to be in fact a manifestation of a *bona fide* infection. Since the latter is limited to a single infectious cycle, it is not surprising that "toxic" signs appear only if the inoculum contains a large number of active virus particles, enough to infect simultaneously a large number of cells.

The type of cells which support the incomplete growth can only be surmised. The manifestations of viral "toxicity"(3) strikingly resemble the acute convulsive seizures seen in infection of mice with a number of neurotropic viruses, *e.g.*, equine encephalomyelitis, and suggest impairment of neuronal elements. By the same token, one may assume that the ability of non-neurotropic influenza viruses to interfere with equine encephalomyelitis and other neurotropic viruses (4) is due to viral activity affecting the same cells. Indeed, recent experiments have suggested that in mouse brains heavily infected with W.E.E. virus the yield of influenza hemagglutinin is lower than in normal mouse brains(22).

Some possible wider implications of the findings suggest themselves. Thus far, no viruses other than the influenza strains mentioned have been tested for their ability to induce the incomplete growth cycle, nor have conclusive tests been done with organs other than the mouse brain. Such tests are in progress with a view toward possible explanation of the "toxicity" of influenza viruses when given by routes other than the intracere-

22. Schlesinger, R. W., unpublished observations.

bral in mice(23) and in rabbits(24). Influenza virus and the other hemagglutinating viruses lend themselves particularly well to this type of investigation, because both, the soluble complement-fixing antigen and the hemagglutinin, can be measured apart from the infectivity as specific phases of viral activity. It is fascinating to speculate upon the possibility that intermediate stages in the reproductive cycle of other viruses may also become recognizable.

Summary. Mouse brain, previously considered as insusceptible to infection with non-neurotropic strains of influenza virus, has been found to support a single cycle of viral reproduction in which hemagglutinin and complement-fixing antigen increase without concomitant rise in infective titer. Rather, the infective titer decreases progressively in mouse brain. The newly formed, incomplete virus is non-infectious, but is produced only if the intracerebral inoculum contains fully infectious virus. The yield of incomplete virus is proportional to the amount of infectious virus inoculated. In mouse brain, as in the allantoic membrane, the constant period preceding a rise in hemagglutinin titer is longer for the Lee than for the PR8 strain. These findings appear to have significance in relation to the "toxicity" of non-neurotropic influenza viruses and to their ability to interfere with unrelated neurotropic viruses, and to the problem of viral adaptation to experimental hosts.

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Absence of Digitalis-like Cardiac Effects in the Action Pattern of Several Simple Lactones.* (17967)

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The cardiac glycosides are currently being subjected to investigative studies aimed at recognition of various relations of chemical structure and biological activity. Interest has centered on the lactone ring moiety and degradation of this component has been described as abolishing the typical digitalis effects(1). Further, various series of the simple lactones have been described from several laboratories as exhibiting positive inotropic effects when tested with cold-blooded hearts(1-10). At the same time, one of these reports by Chen *et al.*(3) presents evidence that a lactone producing digitalis-like effects on frog-heart preparations fails to exhibit such effects when tested on mammalian hearts. This observation made by electrocardiographic recordings in cats has been

* This investigation was supported by a research grant from the National Heart Institute, U. S. Public Health Service.

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8. Giarman, N. J., *J. Pharm. and Exp. Therap.*, 1949, v96, 119.

9. Carlson, R. M., and Seager, L. D., *Fed. Proc.*, 1948, v7, 210.

10. Schueler, F. W., and Hanna, C. C., *Fed. Proc.*, 1950, v9, 313.

confirmed in the present report and has been extended by the application of a method determining the contractile force changes of mammalian hearts *in situ*. This same procedure of characterization has also been applied to other lactones previously described as exhibiting digitalis-like effects in frog-heart preparations. The essential objective of this study has been a determination of the suitability of the biologic characterization methods which may be used in the study of structure-activity relationships of this drug group.

Method. The contractile force of a segment of the right ventricle in open-chest dog preparations was measured according to procedures previously described(11). Factors influencing such determinations have been recently studied and described(12). Concomitant recordings of arterial pressures and electrocardiograph effects were made as before. Results obtained with lactones of the present study were compared against separate characterization studies which have been made under the same conditions with several cardiac glycosides, sympathomimetic amines, veratrine alkaloids and erythrophleum alkaloids. Each of these groups has been found to exhibit distinct and relatively consistent increments in contractile force when administered in favorable dose ranges. In addition to these observations with an *in situ* preparation, experiments were conducted with a modification of the usual Langendorff-Martin arrangement for coronary perfusion of the isolated rabbit heart.

The lactones studied were β,γ-angelica lactone(2-4); l-ascorbic acid(5,6); Ψ-santonin(1,7); the di-lactone of pulvinic acid(8) and the di-lactone, dicumarol(9).

Procedure and results. In 2 experiments, β,γ-angelica lactone was initially administered in doses of 400 mg/kg by intravenous infusion of a 4% aqueous solution over a period of 30 minutes. There were no effects, or slight depression from which recovery was prompt. Subsequent administration of the same doses and of larger doses produced

FIG. 1.

Myocardiograph and arterial pressure tracing. Open-chest dog preparation with Cushny myocardiograph levers attached to anterior wall of right ventricle. Encircled figures represent contractile force in grams determined by spring tension necessary to bring lever stroke to near standstill. Downstroke produced by systolic contraction. Time marker in minutes. The animal had previously received 1200 mg/kg of the lactone in 4 instalments over a total experimental period of 5 hours. During this 5th instalment the cardiac depression was sufficiently marked to require the interruption of the usually attempted 30-minute infusion period.

significant depression of the contractile force and, commonly, the 30 minute infusion procedure had to be discontinued because of marked cardiac depression. In each experiment, total amounts of about 1500 mg/kg were administered over periods of 3½ and 5½ hours. (Fig. 1) Virtually complete recovery occurred and the experiments were subsequently terminated by other means. Systemic arterial pressure changes were not marked or constant in direction. Most frequently the pressure was slightly increased, an effect evidently influenced by the volumes of fluid administered. ECG changes throughout suggested those which might have been obtained with any general depressant.

Several injections of aqueous solutions of the lactone into the perfusion fluid of two isolated rabbit heart preparations consistently produced depression with, usually, prompt recovery to approximately control levels. Varying strengths of solution from 0.5 to 4.0% were used, this highest concentration bringing about complete diastolic standstill with recovery occurring after several minutes. This compound was prepared according to the method of Gilmour(13), observed physical constants being in agreement (b.p. 64-66°

11. Walton, R. P., Jones, H. P., and Leary, J. S., *J. Pharm. and Exp. Therap.*, 1950, v98, 346.

12. Walton, R. P., Cotten, M. deV., Brill, H. H., and Gazes, P. C., *Am. J. Physiol.*, 1950, v161, 489.

13. Gilmour, R., *J. Chem. Soc.*, 1914, v105, 73.

at 15 mm).

L-ascorbic acid was administered in 5% solutions in water by intravenous infusions over periods of 30 minutes. In 3 separate experiments the single doses were 50, 150 and 250 mg/kg respectively. The observations were continued for 3 to 4 hours after the drug was administered. Aside from the fact that the largest dose produced a moderate depression of contractile force (about 20% of that in the control period) there were no significant effects on contractile force, systemic arterial pressure or ECG tracings. It should be noted that the earlier reported cardiotonic actions of these two lactones in frog heart preparations have subsequently been ascribed to the formation of hydrogen peroxide in the solutions used(14-16).

In one experiment Ψ-santonin was administered by intravenous infusion over a period of 30 minutes using a saturated aqueous solution which constituted a dose of about 35 mg/kg. No significant effects were noted. In another experiment, 150 mg/kg in a 20% ethyl alcohol solution was administered in two instalments over a period of 40 minutes. Because of the distinct depression of both systemic arterial pressure and contractile force, the infusion could not be given directly in the usual 30 minute infusion period. The depression was distinctly greater than was obtained from the same amount of ethyl alcohol alone. The condition of the circulation returned to approximately control level 10 minutes after discontinuance of the infusions. No significant ECG changes were noted in either of these 2 experiments. We are obliged to Prof. G. R. Clemo, University of Durham, Newcastle-upon-Tyne, for kindly supplying us with 2 g of this compound.

The di-lactone of pulvinic acid, administered by intravenous infusion to two dogs over a 30-minute period in doses of 20 cc/kg of a saturated aqueous solution (Ringer-Locke), produced no significant changes. Similarly, no significant effects were obtained with doses of 1 and 2 mg/kg administered by immediate intravenous injections of a 1% solution in dioxane. A single injection of 8 mg/kg of the di-lactone of pulvinic acid in the dioxane solution resulted in prompt heart failure. This effect was somewhat greater than that obtained with equivalent volumes of dioxane judged on the basis of a few observations with dioxane alone. In every case, the drug was precipitated as the dioxane entered the saline of the vein cannula system. This di-lactone of pulvinic acid was prepared by the method of Volhard(17) by condensing benzyl cyanide with ethyl oxalate in the presence of metallic sodium to produce oxalyl-bis-benzylcyanide. The oxalyl-bis-benzylcyanide was hydrolysed and condensed to form the di-lactone by heating with three parts of aqueous 60% sulphuric acid. The precipitate was recrystallized from dioxane giving a bright yellow crystalline product agreeing in physical characteristics with recorded data(17).

Dicumarol exhibited no basic similarity to the cardiac glycosides. Unlike the other lactones, however, it did, on occasion, produce marked increments in contractile force of the *in situ* heart preparations. This action has been identified as being due to a general calorigenic action closely related to that produced by dinitrophenol. This study has been reported in abstract(18).

Conclusions. Some relatively simple compounds with the lactone structure have been shown to be lacking in characteristic digitalis-like actions when tested in mammalian heart preparations. Each of these compounds had been previously shown to produce digitalis-like effects in frog heart preparations. The present experiments emphasize the low prediction value of frog-heart preparations in any screening procedure intended for application to mammalian hearts.

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A New Technic for the Continuous Recording of the Cardiac Output.* (17968)

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The output of the heart, or a portion of it, has been measured directly in the anesthetized animal with different types of flowmeters inserted either into the venae cavae(1,2) or the aorta(3,4). Recently Seely and Gregg(5) designed a technic for the measurement of the pulmonary artery outflow by means of an electromagnetic rotameter. The technic described in this paper permits the continuous recording with an electromagnetic rotameter(6,7) of the output of the left ventricle in the anesthetized dog with cerebral circulation intact.

In dogs anesthetized with sodium pentobarbital, the chest is opened through a midsternal incision. Under artificial respiration, the brachiocephalic trunk, a segment of the left subclavian artery close to the aorta, a segment of both common carotid arteries and a segment of the thoracic aorta distal to the left subclavian artery are dissected free. Heparin is then administered, first a dose of 5 mg per kg of body weight, then 3 mg per kg every half hour. As seen in Fig. 1, the aortic end of the left subclavian artery A is first connected to the tubing B leading into the flowmeter (rotameter) C. Then the cephalic end of the right carotid artery D is cannulated and thereby connected to the tubing F coming from the flowmeter C. At this time, the blood flows from the aorta G into the right carotid

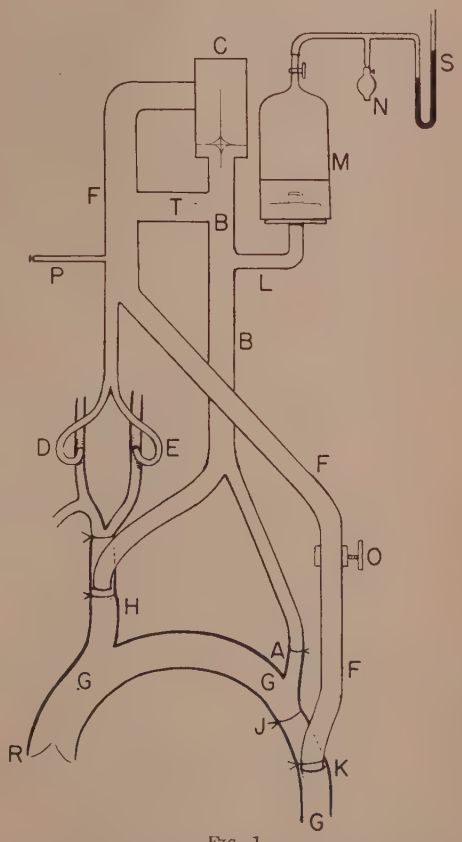

Fig. 1.

Schema of the apparatus used to measure and record the output of the left ventricle. Description in text.

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† Fellows of the New York Heart Assn.

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§ Fellow of the American Heart Assn.

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artery D via the left subclavian artery A, inflow tube B, flowmeter C and outflow tube F. The left common carotid artery E is then also cannulated. At this point, the aortic end of the brachiocephalic trunk H is cannulated and connected to the inflow tube B. The aorta is then ligated at J, just beyond the origin of the left subclavian artery, then cannulated at K and connected to the outflow tube F. When the aorta is ligated at J just before being cannulated, an excessive rise of blood pressure in the aorta and its branches above the ligature at J is prevented by allowing blood to escape via tube L into a buffer reservoir M, the pressure of which is adjusted by a sphygmomanometer bulb N. The amount of blood allowed in the reservoir M is such as to maintain the blood pressure in the aorta and its branches above the ligature at J only slightly above the level of the blood pressure before the ligature of the aorta, the level being read from a mercury manometer S. When the aorta has been cannulated at K, the screw clamp O compressing the outflow tube F is gradually released and blood is slowly pumped back into the dog from the buffer reservoir M. This is done slowly and gradually to prevent any sudden or marked drop in blood pressure in the aorta and its branches above ligature J. When the procedure is completed, the blood flows from the aorta, via the brachiocephalic trunk H and the left subclavian artery A into the inflow tube B and the flowmeter C. From the flowmeter C the blood flows into the common carotids D and E and the descending aorta at K, via the outflow tube F. It can be seen that the blood flowing through the flowmeter is the output of the left ventricle except the coronary blood flow and the amount of blood drained by the few small arteries originating from the aorta between R and J. A tubing, T, which allows shunting of the blood past the rotameter, is clamped when the cardiac output is being measured and is only used to record a zero flow deflection without interfering with the circulation. The buffer reservoir M has also been found useful in damping the pulsation of the flow. The arterial blood pressure is recorded photographically by a Gregg manometer, P. This new technic of continuously recording the output of the left ventricle in the anesthetized dog with cerebral circulation intact has been used successfully in several studies.

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Susceptibilities of Pleuropneumonia-like Organisms to Some Selective Bacteriostatic Agents. (17969)

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(Introduced by Stuart Mudd)

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Although pleuropneumonia-like organisms (PPLO) are occasionally isolated in pure culture from various pathological processes, more often these organisms are present along with bacteria of various kinds. Frequently it is very difficult and sometimes it is impossible to isolate the PPLO in pure culture because they require an enriched medium and an incubation period of a few days. The small size of the colonies of the PPLO compared to that of ordinary bacteria together with the inability to fish off colonies of the PPLO with an inoculating needle contributes to the difficulty of isolating them in pure culture. It would be highly advantageous if some substance could be added to the culture medium which would inhibit bacteria of various kinds but allow the PPLO to grow. The determinations of the susceptibilities of PPLO from human sources to some of the

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TABLE I.
Action of Selective Bacteriostatic Agents on PPLO and Bacteria in Solid Media.

		Basic fuchsin				Crystal violet		Potassium tellurite		
Strains	Control	1:25,000†	1:50,000	1:100,000	1:200,000	1:100,000	1:250,000	1:10,000	1:25,000	1:50,000
07	127*	0	0	30	97	110	114	100	107	103
09	132	0	0	14	117	132	140	133	133	135
36	3	0	0	<1	3	1	2	0	0	2
37	6	<1	3	5	5	2	5	3	5	6
43	5	0	2	4	4	1	5	5	5	5
48	3	0	1	3	3	2	3	1	3	4
60	11	0	1	13	13	0	12	5	10	12
Campo "L"	66	0	13	60	67	63	59	62	58	61

* Numbers indicate the mean colony count in 6 low power microscopic fields.
† Numbers indicate final dilution of selective agent in the media.

TABLE II.
Action of Selective Bacteriostatic Agents on PPLO and Bacteria in Liquid Media.

		Basic fuchsin			Crystal violet	Potassium tellurite	
Strains	Control	1:25,000†	1:50,000	1:100,000	1:100,000	1:10,000	1:50,000
07	>300*	>300	>300	>300	>300	>300	>300
09	>300	106	>300	>300	>300	>300	>300
37	>200	0	>200	>200	>200	>200	>200
48	>200	>200	>200	>200	>200	5	>200
60	>200	>200	>200	>200	>200	0	>200
Campo "L"	>200	2	94	>200	>200	>200	>200

* and † explained in footnotes to Table I.

various chemicals frequently employed in culture media for their selective bacteriostatic action are herein reported because they offer help in overcoming some of the above-mentioned difficulties.

Penicillin is the only antibiotic which has been shown repeatedly(1-4) to be ineffective in inhibiting growth of PPLO. Dienes(2) has pointed out two possible sources of errors in isolating the PPLO resulting from penicillin being present in the culture medium. When bacteria are inhibited by the presence of penicillin in a solid culture medium, the bacterial colonies may be very small and the organisms may swell into round bodies which are indistinguishable from those in colonies of PPLO. The other source of error is that in the presence of penicillin bacteria of several species have been observed to dissociate into the L-type of growth. These instances have been summarized in a previous paper(3). Since penicillin has these disadvantages, substances other than the antibiotics were investigated; namely, basic fuchsin, brilliant green, crystal violet, Nile Blue A, potassium tellurite, sodium azide, and thionin.

Since the bacteriostatic effect of agents may vary in liquid and solid media, both types of media were investigated. For the liquid medium Bacto-heart infusion broth, pH 7.8 was employed. The solid medium was essentially Bacto-heart infusion agar in which the tryptose was replaced by Bacto-peptone. Work in progress and which will be reported later indicates that a medium containing an infusion from beef heart muscle and Bacto-peptone is best for growing the PPLO. To

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2. Dienes, L., PROC. SOC. EXP. BIOL. AND MED., 1947, v64, 165.
3. Leberman, P. R., Smith, P. F., and Morton, H. E., *J. Urol.,* 1950, v64, 167.
4. Hatch, M. H., A symposium on current progress in the study of venereal disease, U. S. Government Printing Office, 1949, 183.

both the liquid and solid media was added ascitic fluid to 25 per cent of the final volume. The various chemicals were added to give the final concentrations as indicated in Tables I and II. For each strain of PPLO a block of agar containing numerous colonies was ground with a glass rod in a test tube containing 5 ml of 2% solution of Bacto-proteose peptone No. 3. After allowing the particles of agar to settle, 0.05 ml of the suspension was transferred to the surface of each plate and was then spread over the surface of the medium with a bent glass rod so as to inoculate the entire surface of the medium uniformly. The plates were incubated aerobically at 37°C for 3 days. The average number of colonies per 6 low power microscopic fields was determined. In the case of liquid media 0.1 ml of the suspension was added to 10 ml of the test medium and this was incubated aerobically for 3 days at 37°C. Since no appreciable amount of turbidity develops when PPLO grow in liquid media, a standard 4 mm platinum loopful of the incubated test cultures was streaked over the surface of a plate of the above-mentioned solid medium without inhibitors. These subculture plates were incubated and examined as described above.

Eight strains of PPLO from humans were tested. Strains 07, 09, and Campo "L" had been propagated on artificial media longer than the remaining 5 strains. Strains 07 and 09 were isolated from the urethra and were received from Johns Hopkins Hospital through the courtesy of I. G. Schaub. Campo "L" was received from the Massachusetts General Hospital by the courtesy of L. Dienes. The remaining 5 strains were isolated by ourselves from patients in the Urology Clinic of the Hospital of the University of Pennsylvania. Strains 36, 37, and 43 were isolated from prostatic secretions, strain 48 from urethral discharge of a male, and strain 60 from a cervical exudate. Eight representative strains of gram-negative and gram-positive organisms were also tested on the same media containing maximum concentrations of the selective agents which did not inhibit PPLO. The other concentrations were unimportant for determining the selectivity of the bacteriostatic agents.

Nile Blue A proved to be too toxic for PPLO to be considered as a selective agent favoring the isolation of these organisms. In the solid medium concentrations of Nile Blue A of 1:100,000 to 1:1,000,000 completely inhibited 7 of the 8 strains of PPLO and the eighth strain, Campo "L", grew slightly in the lesser concentration of the dye. Essentially the same results were obtained with the same concentrations of the dye in liquid media. It has occasionally been reported that colonies of PPLO have been detected on media employed for isolating the gonococcus from clinical material. From these results it is obvious that those media employed for cultivating the gonococcus which contain Nile Blue A are unsuitable for detecting PPLO.

Thionin was another dye which proved to be too toxic for PPLO to be employed as a selective agent. Concentrations of 1:10,000 and 1:25,000 were completely inhibitory in both the solid and liquid media. A concentration of 1:200,000 was toxic for some of the strains.

Brilliant Green inhibited completely the growth of PPLO on the solid medium in concentrations of 1:25,000 to 1:200,000. In the liquid medium in concentrations of 1:100,000 and 1:200,000, the PPLO and the gram-negative bacteria were not inhibited but the gram-positive organisms were inhibited completely with the exception of *C. xerose* which was uninhibited.

The greatest concentration of sodium azide in the solid medium which did not inhibit growth of the PPLO was 1:25,000 and at this concentration 3 of the 4 gram-positive organisms were uninhibited.

Of the substances tested basic fuchsin, crystal violet, and potassium tellurite gave the best results in selectively inhibiting either the gram-negative or gram-positive organisms while at the same time not inhibiting the PPLO. In the solid medium basic fuchsin, 1:200,000, and crystal violet, 1:250,000, did not inhibit the 4 gram-negative bacteria, *E. coli, P. vulgaris, A. aerogenes,* and *Ps. aeruginosa,* but did inhibit the 4 gram-positive organisms, *S. aureus, S. albus, St. fecalis* and

C. xerose. The 1:50,000 concentration of potassium tellurite inhibited the 4 gram-negative bacteria but did not inhibit the 4 gram-positive organisms. Usually greater concentrations of the substances were required to produce the same effect in the liquid medium; basic fuchsin, 1:100,000, and crystal violet, 1:100,000 inhibited the 4 gram-positive organisms but permitted growth of the 4 gram-negative bacteria and the PPLO. Potassium tellurite, 1:50,000, inhibited *E. coli* and *A. aerogenes* but not *P. vulgaris* and *Ps. aeruginosa* among the gram-negative bacteria and in addition inhibited the gram-positive organisms except for *C. xerose.*

Discussion. To our knowledge the only other work on the susceptibility of PPLO to bacteriostatic agents is that of Edward(5). Whereas he observed that neither of his 2 strains isolated from mice was inhibited by 1:5,000 sodium azide, we observed that 5 of our 8 test strains were inhibited. The 3 of our strains not inhibited by 1:5,000 sodium azide were strains which had been on artificial media for some time. Our strains from humans, even the recently isolated strains, appear to be slightly more resistant to potassium tellurite and crystal violet than were the two mouse strains tested by Edward. Like Edward, we found that PPLO were inhibited by brilliant green in 1:100,000 concentration. In addition, Edward investigated thallium acetate but it appears to offer no advantages over potassium tellurite which is commonly used to inhibit gram-negative organisms.

Summary. Strikingly, the PPLO do not behave either as gram-positive or gram-negative microorganisms in that they are not inhibited on solid medium by a 1:250,000 dilution of crystal violet which normally inhibits gram-positive microorganisms nor by a 1:50,000 dilution of potassium tellurite which normally inhibits gram-negative organisms. By being able to inhibit both gram-positive and gram-negative microorganisms the isolation of PPLO from mixed cultures can be greatly facilitated. Generally, strains which have been cultivated on artificial media for some time are more resistant to the bacteriostatic substances than are the recently isolated strains.

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Production, Purification, and Some Properties of *Clostridium histolyticum* Collagenase.* (17970)

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Jennison(1) reported that *Clostridium histolyticum* produced a proteinase that was able to digest "native" collagen. In a previous communication from this laboratory(2) the activity of *Cl. histolyticum* filtrates in the degradation of collagen was confirmed, as well as the lack of activity of other proteinases on unmodified collagen.

In this communication a reproducible procedure will be described for the isolation and partial purification of a proteinase(s) from *Cl. histolyticum* which is very active in the solubilization of native collagen. A method will be described wherein filtrates with little or no lethal toxin but adequate "collagenase" activity may be produced in large batches. The final product purified by the method to be described is 400 times as active per unit nitrogen as the original filtrate. The separation of a proteolytic enzyme which has no collagenase activity and which is activated

* This work was done under a contract between the U. S. Army and the University of Cincinnati.

1. Jennison, M. W., *J. Bact.,* 1945, v50, 349.

2. Neuman, R. E., and Tytell, A. A., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 409.

by Fe^{++} and cysteine (gelatin substrate) is described. This enzyme has been described previously(3,4).

Experimental. Stock and seed cultures. The strain of *Cl. histolyticum* used in these studies was CHT from the stock collection in this laboratory. The stock medium was prepared as previously described for *Cl. perfringens*(5). In carrying this culture on stock medium, no glucose was used. Just before transfer, 1 ml of a sterile sodium thioglycolate solution (4 mg) was added to each tube. The stock cultures were incubated at 37°C for 16-18 hours. Seed cultures were incubated for 8 hours.

Medium for collagenase production. Casein digest and medium were prepared as previously described(5). The bottles containing 3 liters of medium were steam sterilized at 15 lb. for 1 hour and cooled to approximately 38-40°C in running tap water. A sterile solution (75 ml) containing 3 g of sodium thioglycolate was added to each bottle immediately prior to incubation with 40 ml of seed culture. The bottles were then placed in a 37°C water bath for 18 hours.

Filtration. The cultures were cleared of most of the organisms by filtration with suction through a layer (1 cm) of supercel (Johns Manville Co.). Clear, sparkling filtrates suitable for the purification procedure were obtained by filtration through 12 inch Berkefeld "N" candles. The filtrates were tested for toxicity by the intravenous route (lethal toxin) in mice (16-18 g) and usually assayed at 1 to 2 LD_{50} per ml.

Dialysis. The filtrates were poured into Visking casing (1⅝″) and dialyzed against cooled running tap water (average temperature 15°C) in a continuous rocking dialyzer for 17-18 hours. If the dialysis was interrupted the sacks were placed in distilled water in the cold room at 4-6°C.

Precipitation with methanol. To each 1500 ml of dialyzed filtrate at 0°C, 1000 ml of methanol (pre-cooled) was added with constant stirring. The precipitate was allowed to form and flocculate for 24 hours during which time the precipitate settled to the bottom of the container. The supernatant fluid was removed by careful siphoning and the precipitate collected in a refrigerated centrifuge.

Precipitation with ammonium sulphate. The methanol precipitate from 1500 ml of dialyzed filtrate was taken up in 40 ml of water, and 20 ml of 0.1 M phosphate buffer at pH 7.2 was added. This yielded a clear pigmented solution. The solution was brought to 75% saturation by the addition of 180 ml of saturated ammonium sulphate at room temperature. The precipitate was allowed to form at room temperature for 24 hours before it was collected by centrifugation in a refrigerated centrifuge. This precipitate was then dissolved in 20 ml of distilled water. The cloudy solution was centrifuged in a refrigerated centrifuge to remove insoluble pigmented material which was discarded. The supernatant was then dried by lyophilization to a clean, white, easily soluble powder.

Measurement of collagenase activity. The activity of enzyme preparations was measured by the release of soluble nitrogen from collagen. The activity was expressed as mg collagen (N x 5.85) solubilized per mg enzyme nitrogen in 18 hours from 100 mg beef tendon collagen† at 37°C. The pH was 7.2; buffer 0.1 M phosphate. A typical experimental batch of 6 liters carried through the purification procedure is shown in Table I. Twelve such batches have been carried to this point with little or no variation.

Results. pH activity curve. The activity of *Cl. histolyticum* collagenase was measured in phosphate buffers from pH 4.6 to 8.9. The greatest activity was attained at pH 7.6 under the conditions of these experiments.

Heat inactivation. At an enzyme concentration of 4 μg nitrogen per ml the collagenase activity was destroyed to the extent of 98.5% in 20 minutes at 50°C. It is of interest to

3. Kocholaty, W., and Krejci, L. E., *Arch. Biochem.*, 1948, v18, 1.

4. van Heyningen, W. E., *Biochem. J.*, 1940, v34, 1540.

5. Boyd, M. J., Logan, M. A., and Tytell, A. A., *J. Biol. Chem.*, 1948, v174, 1013.

† This preparation is described in(2).

TABLE I.
Typical Data* on Enzyme Purification Experiment.

Preparations	mg collagen dissolved per mg N	Recovery % total original activity
Original filtrate	100	
Step 1—Dialyzed filtrate	636	—
" 2—Methanol ppt.	7430	58
" 3—$(NH_4)_2SO_4$ ppt.	38000	61.5

* Data from one of 12 batches of 6 liters each.

TABLE II.
Effect of Normal Sera, Specific Antisera, and Soy Bean Trypsin Inhibitor on *Cl. histolyticum* Collagenase. Substrate level 100 mg beef tendon collagen. Enzyme level 1 μg nitrogen. Incubation at 37°C for 18 hr.

Serum	Amt test, ml	Inhibition, %
Normal horse I*	.05	11
" " I*	.10	30
" " II*	.05	5
" " II*	.10	21
Horse antiserum I*	.001	0
(100 units antitoxin/ml)	.005	100
Horse antiserum II*	.001	91
(350 units antitoxin/ml)	.005	97
Normal guinea pig serum	.05	10
" " " "	.10	31
" rabbit serum	.05	0
" " "	.10	0
" " "	.20	23.5
Soy bean trypsin inhibitor† (crystalline)	150 μg	0
	300 μg	0

* Obtained through the courtesy of Dr. Irvin S. Danielson, Lederle Laboratories Division, American Cyanamid Co.

† Obtained through the courtesy of Dr. M. Kunitz, Rockefeller Institute.

note that this heated preparation retained 30% of its original activity on a casein substrate.

Effect of normal sera, specific antisera, and crystalline soy bean trypsin inhibitor. Normal horse, guinea pig, and rabbit sera were tested and showed only partial inhibition of collagenase activity at relatively high concentrations. Specific antisera inhibited collagenase activity at relatively low concentrations. The efficacy of the specific antisera was not proportional to the antitoxin potency. Under the conditions of the experiments collagenase activity was not inhibited by the crystalline soy bean trypsin inhibitor. A typical experiment is shown in Table II.

Effect of Fe^{++}, cysteine. Ferrous iron (as ferrous sulphate) and cysteine alone or together did not enhance collagenase activity (Table III). In fact in most instances these substances inhibited collagenase activity to a significant degree. These results are in agreement with those of Maschmann(6) but not in accord with the properties of the proteinase isolated by Kocholaty and Krejci(3). This discrepancy is explained by the separation of a second proteolytic enzyme from *Cl. histolyticum* filtrates as will be shown in a later section. These findings are in agreement with those of van Heyningen(4).

TABLE III.
Inhibitory Effect of Fe^{++} and Cysteine on *Cl. histolyticum* Collagenase.

Substance	mM	Inhibition, %
Fe^{++}	.01	53
"	.10	79
Cysteine	.01	17
"	.10	0
Fe^{++} and cysteine	.01 each	18.7
" " "	.10 "	40.7

Action of specific inhibiting substances. The collagenase activity was inhibited by or-

6. Maschmann, E., *Biochem. Z.*, 1937, v295, 391.

TABLE IV.
Effect of Several Inhibitors on *Cl. histolyticum* Collagenase.

Substance	mM	Inhibition, %
Mercuric chloride	.05	100
Phenylmercuric acetate	.05	87
p-Chloromercuribenzoate	.008	100
Copper sulfate	.01	82.5
Formaldehyde	.30	100

ganic mercurials, copper sulphate, and formaldehyde in relatively low concentrations (Table IV). More extensive studies on this phase of the problem are in progress.

Separation of a proteolytic enzyme activated by Fe^{++} and cysteine. By precipitation from *undialyzed* filtrates with 66% methanol at 0°C an enzyme preparation was obtained which was active on gelatin but not on collagen. This preparation was further purified by precipitation from 0.05 M acetate buffer at pH 4.6. The product was activated by Fe^{++} and cysteine and its properties more closely resembled the proteinase described by Kocholaty and Krejci(3) and van Heyningen (4).

Summary. A consistently reproducible method is described for producing *Clostridium histolyticum* filtrates with good collagenase activity and little or no toxin. The proteolytic enzyme which attacks collagen has been purified 400 times based on activity per unit of nitrogen. The "collagenase" activity is not enhanced by Fe^{++} and cysteine. The collagenase activity is inhibited by specific *Cl. histolyticum* antisera in low concentrations. This inhibition is not proportional to the antitoxic value of the antiserum. Normal rabbit, guinea pig, and horse serum in high concentrations partially inhibit collagenase activity. Collagenase is not inhibited by crystalline soy bean trypsin inhibitor. Collagenase activity is inhibited by reagents supposedly specific for amino groups and sulphydryl groups. It is also sensitive to heavy metals. Collagenase activity is destroyed at 50°C in dilute solutions of enzyme. A proteolytic enzyme, activated by Fe^{++} and cysteine, active on gelatin and not active on collagen is also present in *Cl. histolyticum* filtrates.

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Pteroylglutamic Acid-like Effect of Dehydroisoandrosterone on Growth of Certain Microorganisms.* (17971)

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During routine testing of a number of compounds for ability to reverse aminopterin inhibition of *Streptococcus faecalis* it was found that dehydroisoandrosterone acetate† was especially active. Further trials revealed that this steroid has pteroylglutamic acid (PGA) like activity for both *S. faecalis* and *Lactobacillus casei* and is capable of stimulating *Escherichia coli* in the presence of inhibiting levels of sulfanilamide. Several other steroids and 2 carcinogens were tested and a few found to have very slight PGA-like activity. Although the activity was small when compared with that of pteroylglutamic acid the ability of compounds such as sex hormones and carcinogens or their derivatives to stimulate cell division in a manner similar to that of pteroylglutamic acid could hardly fail to have great biological significance. Further experiments on steroids are under way. The data presented here are representative of a large number of tests made to determine the

* Research paper No. 906, Journal Series, University of Arkansas.

† We would like to express our appreciation to the Schering Corporation for furnishing us with the dehydroisoandrosterone acetate used in this experimental work.

TABLE I.
Pteroylglutamic Acid-like Effect of Dehydroisoandrosterone Acetate (DIA) on *Streptococcus faecalis* and on *Lactobacillus casei*.

Organism	Additions to medium, γ/10 ml	Photometric density 16 hr	Photometric density 40 hr	ml 0.1 N NaOH, 46 hr
S. faecalis	PGA			
	0	.02	.03	0.8
	.001	.17	.21	4.1
	.005	.29	.32	4.8
	DIA			
	20	.17	.29	5.1
	100	.24	.35	5.1
	400	.38	.55	5.3
L. casei	PGA			72 hr
	0.			2.2
	.0004			5.8
	.001			7.5
	.005			8.9
	DIA			
	.25			2.9
	2.5			6.2
	10.			8.5
	300.			10.1

nature of the growth stimulating activity of dehydroisoandrosterone. It should be recalled that Barton-Wright *et al.*(1) found folic acid-like activity in chloroform extracts of liver several years ago.

Experimental. The organisms used for the present study were *Streptococcus faecalis* (American Type Culture Collection No. 8043), *Lactobacillus casei,* and a laboratory strain of *Escherichia coli.* The medium used for *S. faecalis* and *L. casei* was similar to that of Mitchell and Snell(2). For convenience the same medium with the omission of all vitamins except pantothenic acid was used for *Escherichia coli.* All organisms were incubated at 32°C for periods of time ranging from 16 to 72 hours. Titration of the lactic acid produced by *S. faecalis* or by *L. casei* was made at the times indicated in Table I. Turbidity measurements were made at intervals on a Coleman Junior spectrophotometer and recorded.

Results.‡ The ability of dehydroisoandrosterone acetate to stimulate the growth and acid production of *Streptococcus faecalis* and *Lactobacillus casei* is shown by typical results recorded in Table I. Results with PGA are also given for comparison. It may be seen that the steroid is required in amounts about 20,000 times as great as PGA to produce one-half maximum growth at 16 hours. However, this ratio differs with the incubation times and with the species of organism used. These data, as well as those in Table II, indicate that the activity of the steroid is not due to any possible contamination with PGA. Additional assurance that PGA contamination was not responsible for the activity was obtained by refluxing the steroid for 8 hours in 1 N HCl. This treatment did not materially affect the activity although PGA is almost completely destroyed by such a procedure. After 36 sub-cultures on dehydroisoandrosterone acetate alone *Streptococcus faecalis* still required PGA or dehydroisoandrosterone although the organism seemed to adjust itself to lower concentrations

1. Barton-Wright, E. C., Emery, W. B., and Robinson, F. A., *Biochem. J.,* 1945, v39, 334.

2. Mitchell, H. K., and Snell, E. E., *Univ. Texas Pub. No. 4137,* 1941, 36.

‡ At our request, Dr. E. L. R. Stokstad very kindly checked our results with dehydroisoandrosterone acetate obtained by him from the Schering Corporation. According to a personal communication he obtained activity similar to that reported here.

TABLE II.
Growth Stimulation of Various Organisms by Dehydroisoandrosterone Acetate (DIA) and PGA in the Presence of Inhibitors.

Organism and inhibitor	Additions to medium, γ/10 ml	Photometric density 16 hr	Photometric density 40 hr
Streptococcus faecalis	PGA		
Pteroylaspartic acid			
2 γ/10 ml	0	0	0
,,	.001	0	0
,,	.005*	0	0
	DIA		
,,	20	.07	.16
,,	50	.13	.26
,,	300	.41	.51
4 amino PGA	PGA		
.05 γ/10 ml	0	.08	.11
,,	.001	.11	.11
,,	.005	.15	.15
	DIA		
,,	20	.07	.12
,,	100	.11	.22
,,	300	.28	.48
Escherichia coli	PGA		
Sulfanilamide			
16 mg/10 ml	0	.04	.15
,,	.001	.08	.24
,,	.005	.09	.26
	DIA		
,,	20	.17	.26
,,	50	.11	.30
,,	400	.08	.22
	Thymine		
,,	500	.19	.24

* This quantity of PGA in the presence of inhibitor gave good growth (P.D. = 0.3) at 112 hours.

of dehydroisoandrosterone.

The mode of action of the sex hormone is not similar to that of thymine. This may be seen by examination of the data in Table II. The inhibition due to 4 amino pteroylglutamic acid or to pteroylaspartic acid appeared to be reversed competitively by dehydroisoandrosterone. Thymine reverses this type of inhibition non-competitively(3).

The action of the dehydroisoandrosterone in promoting growth of the test organisms closely simulates the activity of PGA but some differences seem to exist. At optimum concentration and with the medium used the steroid appeared to stimulate somewhat more growth with *Streptococcus faecalis* than was obtainable with PGA at any concentration. On the other hand the organisms tested did not reach maximum growth as quickly with dehydroisoandrosterone acetate as with PGA. It is not unlikely that the substitution for PGA occurs better for certain functions of the vitamin than for others. However, there is also a possibility that in the presence of the hormone the organisms are stimulated to produce PGA.

Summary. Dehydroisoandrosterone acetate was found to have pteroylglutamic acid-

3. Hutchings, B. L., Monat, J. H., Oleson, J. J., Stokstad, E. L. R., Boothe, J. H., Waller, C. W., Angier, R. B., Semb, J., and SubbaRow, Y., *J. Biol. Chem.*, 1947, v170, 1323.

like activity for *Streptococcus faecalis, Lactobacillus casei* and sulfonamide-treated *Escherichia coli.* The activity of the steroid differs from that of thymine but is of the same order of magnitude.

We are indebted to Lederle Laboratories Division, American Cyanimid Company for the supplies of pteroylglutamic acid and pteroylglutamic acid antagonists used in this study.

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On Hemolysis Mediated by Non-Erythrocytic Antigens, Their Homologous Antibodies and Complement. (17972)

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Kondo(1) observed hemolysis in mixtures of sheep erythrocytes, suspensions of *Serratia marcescens,* and fresh guinea pig serum. He attributed lysis to the enhancement of bacterial hemolysin by guinea pig complement. A recent brief note by Fisher and Keogh(2) described the lysis by complement of erythrocytes which had adsorbed bacterial components and anti-bacterial antibodies. The observations of these authors have been extended in the present report.

Materials and methods. (1) *Antigens.* Trichloroacetic extracts(3) were prepared from *Salmonella typhosa* O 901 and from *Escherichia coli,* strain H. After dialysis against water, the extracts were concentrated to contain approximately 0.5 mg solids per ml, sodium chloride was added to obtain isotonicity, the reaction was adjusted to pH 7, and sterility was attained by Seitz filtration. In another experiment human plasma, in place of bacterial antigens, was used.

(2) *Antisera and complement.* Seitz filtered, heat-inactivated immune sera from four rabbits were employed. One of each of the immune sera was prepared by immunization with *E. coli, S. typhosa,* human serum, and washed sheep erythrocytes. For the detection of rabbit serum adsorbed on the surface of erythrocytes, a chicken immune serum against rabbit euglobulins, absorbed with rabbit serum albumin and pseudoglobulin, was used. Aliquots of immune sera, employed in some experiments, were freed of normal hemagglutinins by double absorption at 37°C (two 15 minute periods) with 0.5 ml packed erythrocytes for each ml of serum. The fresh pooled serum from at least 3 normal guinea pigs, diluted 10-fold in saline, served as complement.

(3) *Erythrocytes* and *"sensitization."* Erythrocytes from sheep blood preserved in Alsever's solution, rabbit cells from fresh defibrinated blood, and human type O cells from citrated blood were used. The cells were usually washed twice in saline, and a 1% suspension was then prepared, using as suspending fluid (a) antigen solution or immune serum dilution, respectively, and (b) salt solution (controls).* With occasional agitation, these cell suspensions were incubated one hour at 37°C, the cells were then washed twice in saline, and 1% cell suspensions in saline were prepared of both "sensitized" and "non-sensitized" (control) cells.

(4) *Hemolysis titration.* Twofold serial dilutions of antigen solution or of immune serum, respectively, in 0.2 ml amounts, were prepared in duplicate. To both series of tubes were then added 0.2 ml amounts of a 1:10 dilution of complement. One series of tubes received 0.2 ml amounts of 1% sensitized

1. Kondo, S., *Z. Immunitäts.,* 1923, v36, 76.

2. Fisher, S., and Keogh, E. V., *Nature,* 1950, v165, 248.

3. Boivin, A., and Mesrobeanu, L., *C. R. Soc. Biol.,* 1933, v112, 76.

* Cells which were exposed to solutions of bacterial antigens or to serum proteins in the manner just described will be referred to as sensitized cells.

TABLE I.
Effect of Complement Upon Antigen-treated Erythrocytes in the Presence of Non-Hemolytic Corresponding Immune Sera.

Immune serum employed	Designation of erythrocytes used	Reciprocal of final serum dilution									Serum control	Complement control	Cell control
		50	100	200	400	800	1600	3200	6400	12800			
Rabbit anti-*E. coli* serum	A	4	4	4	4	4	3	3	2	0	0	0	0
(Rabbit No. 1)	B	4	4	3	1	0	0	0	0	0	0	0	0
	C	4	4	4	3	1	0	0	0	0	0	0	0
	D	3	2	0	0	0	0	0	0	0	0	0	0
	G	0	0	0	0	0	0	0	0	0	0	0	0
	H	0	0	0	0	0	0	0	0	0	0	0	0
Rabbit anti-*E. coli* serum	A	4	4	4	4	4	3	2	1	0	0	0	0
(Rabbit No. 1)	D	0	0	0	0	0	0	0	0	0	0	0	0
Absorbed with sheep erythrocytes	E	4	4	3	2	1	1	0	0	0	0	0	0
Rabbit anti-*S. typhosa* serum	E	4	4	4	4	3	2	1	0	0	0	0	0
(Rabbit No. 2)	A	3	1	1	0	0	0	0	0	0	0	0	0
	D	0	0	0	0	0	0	0	0	0	0	0	0
Rabbit serum vs. human serum	F	2	4	4	4	4	4	3	1	0	0	0	0
from type O donor	G	0	0	0	0	0	0	0	0	0	0	0	0

A = Sheep erythrocytes, sensitized with *E. coli* extract.
B = Human type O erythrocytes, sensitized with *E. coli* extract.
C = Rabbit erythrocytes, sensitized with *E. coli* extract.
D = Sheep erythrocytes, not sensitized (control cells).
E = Sheep erythrocytes, sensitized with *S. typhosa* extract.
F = Rabbit erythrocytes, sensitized with human plasma from type A donor.
G = Rabbit erythrocytes, not sensitized (control cells).
H = Human type O erythrocytes, not sensitized (control cells).
4 = Complete lysis; 0 = No lysis; 3, 2, 1 = Degrees of incomplete lysis. Results were read after 1 hr incubation at 37°C.

cells, the other series equal amounts of non-sensitized cells. A serum or antigen control tube, in which complement was replaced by an equal volume of saline, a complement control tube, in which serum or antigen was replaced by saline, and a cell control tube containing erythrocytes and saline, were also set up. The total volume in each tube was brought to 1 ml by the addition of saline. The results were read after one hour incubation at 37°C.

Results. (1) A summary of findings pertaining to the lysis of erythrocytes sensitized with different antigens upon exposure to the corresponding immune sera, and complement is presented in Table I. It may be seen that sensitization of red cells was accomplished by human plasma as well as by bacterial extracts. Further, the results show that sensitized cells were lysed even in high dilutions of the immune sera specific for the sensitizing agent (1:400 to 1:12800), and that removal of normal hemagglutinins from such sera failed to depress the hemolytic activities against sensitized cells significantly, while hemolysis of non-sensitized cells was completely abolished. No lysis occurred when either complement or immune serum, or both reagents were omitted. An antigenic relationship between *E. coli,* strain H, and *S. typhosa* O 901, evident from cross-agglutination,† expressed itself in the lytic action of the anti-*S. typhosa* serum on erythrocytes sensitized with *E. coli* extract (1:200), and by the lysis of red cells sensitized with *S. typhosa* extract in the presence of anti-*E. coli* serum (1:1600). Red cells sensitized with the bacterial extracts occasionally were darkened, and tended to clump. To ascertain whether these noticeable changes in the erythrocytes could cause increased sensitivity toward lysis by complement, a 2% suspension of such cells was prepared in saline, and a routine complement titration(4) was carried out, using two units of amboceptor. Both normally appearing, non-sensitized cells, and discolored sensitized cells were equally sensitive to complement. (0.2 ml of a 1:40 dilution of guinea pig serum was the least amount that caused complete lysis in the presence of 2 units of amboceptor).‡

(2) Attempts were made to reverse the process by sensitizing erythrocytes with non-hemolytic immune sera, and to induce lysis by adding such cell suspensions to serial dilutions of the corresponding antigens in the presence of complement. The available rabbit immune sera proved to be unsuitable for the sensitization of sheep cells, since dilutions smaller than 1:10 contained sufficient normal anti-sheep antibody to induce lysis of these cells when complement was added. Recourse was therefore taken to the use of rabbit erythrocytes. Red cells from the defibrinated blood of two rabbits were washed in saline, exposed (in 1% concentration) to a 1:4 dilution of rabbit anti-*E. coli* immune serum for one hour at 37°C, washed once, made up to a 1% suspension in saline, and then added in the usual manner to serial dilutions (from 1:10 to 1:25600) of the *E. coli* extract, in the presence of complement. No lysis ensued. Upon overnight storage of the test in the ice-box, hemagglutination was evident in the tubes containing sensitized cells and extract dilutions from 1:10 to 1:640. Sensitized cells were not agglutinated by the higher extract dilutions, or in the absence of bacterial extract. Non-sensitized cells were not agglutinated.

Since agglutination, but not lysis, had occurred in the previous experiment, an attempt was made to test the efficacy of sheep erythrocyte sensitization with a 1:4 dilution of the *E. coli* antiserum (absorbed with sheep red cells). Aliquots of 0.3 ml of a 1% suspension of sensitized cells, and equal volumes of a similar suspension of non-sensitized erythrocytes, were added to 0.3 ml volumes of serial

†In slide agglutination tests(5), using suspensions of living cells, the anti-*S. typhosa* serum still agglutinated *E. coli* when diluted 1:25, and the anti-*E. coli* serum still agglutinated *S. typhosa* when diluted 1:5.

4. Boyd, W. C., Fundamentals of Immunology, Interscience Publishers, Inc., New York, 1947.

‡ Similarly discolored sheep cells, obtained by treating 5 ml of a 1% suspension in saline with 0.25 ml of a commercial 3% solution of H_2O_2 for 5 minutes at 37°C, proved to be more sensitive to a sample of fresh guinea pig serum which possessed normal sheep cell agglutinins than untreated cells. Untreated cells were lysed in dilutions less than 1:5; while treated cell were lysed even by a 1:20 dilution of the guinea pig serum.

PROTOCOL AND TABLE II.

Fixation of Complement by Sensitized Erythrocytes in the Presence of Corresponding Antigens or Antisera, Respectively.

1	2	3	4	5	6	
Tube	0.2 ml of 1% erythrocyte suspensions	*E. coli* antigen solution (0.2 ml)	*E. coli* antiserum* dilution (0.2 ml)	G. pig complement (1:5 dilution) ml	Saline ml	
A	Sensitized with *E. coli* antiserum*	1:8	—	.2	.4	Incubated for 1 hr in ice bath, then centrifuged and supernate decanted
B	”	1:16	—	.2	.4	
C	”	Saline	—	.2	.4	
D	Sensitized with *E. coli* antigen dil'n	—	1:10	.2	.4	
E	”	—	1:40	.2	.4	
F	”	—	Saline	.2	.4	

7	8	Supernates 9									Sedimented cells 10		11
		Aliquots of 0.15 ml to 0.50 ml of diluted supernates (increments of 0.05 ml) were added to 0.2 ml fresh 2% sheep erythrocyte suspension + 2 units amboceptor incubated 1 hr at 37°C											
Tube	Saline, ml	0.15	0.20	0.25	0.30	0.35	0.40	0.45	0.50	Saline	Saline, ml		Degree of hemolysis
		Degree of hemolysis											
A	2	0	1	1	3	3	4	4	4	0	1	Incubated	0
B	2	0	1	1	2	3	4	4	4	0	1	1 hr	0
C	2	0	1	2	3	4	4	4	4	0	1	at	0
D	2	0	0	0	0	1	2	2	2	0	1	37°C	4
E	2	0	0	0	1	1	3	3	4	0	1		4
F	2	0	1	1	2	4	4	4	4	0	1		0

Key to symbols = Same as in Table I.

* = The antiserum had previously been absorbed with sheep erythrocytes.

twofold dilutions of a chicken anti-rabbit euglobulin serum (absorbed with sheep erythrocytes). After 1 hour at 37°C, and overnight incubation in the icebox, sensitized erythrocytes were agglutinated by chicken serum dilutions up to 1:160, while non-sensitized erythrocytes were agglutinated in dilutions up to 1:20 only.

In view of this evidence of actual adsorption of rabbit euglobulins onto the surface of sheep erythrocytes, it now appeared desirable to investigate whether at least some of the adsorbed euglobulins were *E. coli* agglutinins. Serial twofold dilutions of the *E. coli* antiserum used in sensitizing sheep erythrocytes, and similar dilutions of a sample of the same serum which had not been employed in the sensitization of the sheep erythrocytes, were tested for *E. coli* agglutinins. The results of the agglutination tests(5) showed that a reduction in *E. coli* agglutination titer from 1:1024 (prior to its use for sheep erythrocyte sensitization) to 1:256 (after sheep cell sensitization) had occurred.

Since erythrocytes sensitized by their exposure to *E. coli* antiserum had apparently absorbed some *E. coli* agglutinins, as shown by the results of the previous experiments and by the agglutination of such cells by *E. coli* antigen, it remained to be seen why lysis of such cells did not take place in the presence of *E. coli* antigen upon addition of complement. In order to determine whether the sensitized erythrocytes combined with complement at all in the presence of *E. coli* antigen, a complement-fixation test was carried out by the method outlined in Protocol and Table II.

It may be seen from column 9 of the table that complement was fixed in appreciable quantities only by those sheep cells which had

5. Edwards, P. R., and Bruner, D. W., Serological Identification of Salmonella Cultures, University of Kentucky Agricultural Experiment Station, Circular 54, 1942.

been sensitized with *E. coli* antigen and which subsequently were exposed to *E. coli* antiserum (tubes D and E). Fixation of complement by these cells during the incubation in the ice bath was followed by their lysis on subsequent exposure to 37°C (Column 11, tubes D and E). Sheep cells which had been sensitized with *E. coli* antiserum failed to fix significant amounts of complement when they were exposed to dilutions of *E. coli* antigen solution (Column 9, tubes A and B). Subsequent incubation of these cells at 37°C failed to result in their lysis. (Column 11, tubes A and B).

Summary. The data presented in this paper confirm and extend the recent observations of Fisher and Keogh(2). Erythrocytes from sheep, rabbit, and man, which have adsorbed extracts from *E. coli* and *Salmonella typhosa*, or human plasma, are lysed in the presence of complement and of corresponding immune sera for the adsorbed antigens. Evidence for the specificity of the reaction has been presented. Neither appreciable complement fixation, nor lysis occurred when erythrocytes first sensitized with non-hemolytic immune sera were then exposed to complement and the corresponding antigens.

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A Direct Relationship between Indoleacetic Acid Effects on Growth and Reducing Sugar in Tobacco Tissue.* (17973)

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Numerous attempts have been made to relate the effects of auxin (indole-3-acetic acid (I.A.A.); naphthalene acetic acid; and related compounds) on growth of plants with specific changes in metabolism and cellular constituents. In the case of toxic effects of 2,4-dichlorophenoxyacetic acid (2,4-D) decreases or temporary rises and subsequent depletion of available carbohydrates have been reported(1-4). An increase in reducing capacity in tissues, measured by staining, has been obtained from treatments of bean stem cultures with 2,4-D(5).

Recently Christiansen and Thimann(6) have demonstrated a decrease in reducing sugar in *Pisum* stem sections and *Avena* coleoptile sections grown for short periods of time in solutions of various compositions with auxin concentrations which promote growth. However, they report a similar decrease in controls without I.A.A. and even greater decreases of sugar from the addition of respiratory inhibitors, which completely stop growth, such as iodoacetate, arsenite, and fluoride. In general, tests on changes in composition associated with auxin treatments which promote growth have been carried out in short time experiments under conditions leading to net decreases in dry weight of the tissues, and where their continued growth was precluded. It was of interest, therefore, to carry out analyses for changes in cell constituents in tissues supplied with I.A.A. in media adequate for continuous growth over periods of several months. The experiments reported here, with tobacco stem segments

* This project is supported in part by the University Committee, on funds from the Wisconsin Alumni Research Foundation, and in part by a grant in aid from the Cancer Society upon recommendation of the Committee on Growth of The National Research Council.

1. Mitchell, J. W., and Whitehead, M. R., *Bot. Gaz.*, 1940, v101, 668.
2. Mitchell, J. W. and Brown, J. W., *Bot. Gaz.*, 1945, v107, 120.
3. Sell, H. M., Luecke, R. W., Taylor, B. M. and Hamner, C. L., *Plant Physiol.*, 1949, v24, 295.
4. Weller, L. E., Luecke, R. W., Hamner, C. L., and Sell, H. M., *Plant Physiol.*, 1950, v25, 289.
5. Gall, H. J. F., *Bot. Gaz.*, 1948, v110, 319.
6. Christiansen, G. L., and Thimann, K. V., *Arch. Biochem.*, 1950, v26, 230.

grown *in vitro*, show that under these conditions I.A.A. added to the medium markedly increases the tissue concentrations of reducing sugars. This effect is a function of the I.A.A. concentration and closely parallels its effect on increasing the growth of the tissues.

Materials and methods. Stem segments obtained from young internodes of tobacco (Wisconsin No. 38) grown in the green house were sterilized and cultured as described by Skoog and Tsui(7) on White's nutrient medium with 2% sucrose and 0.7% agar. I.A.A. was added to the medium in concentrations of 0, 0.01, 0.1, and 10.0 mg/l and the pH was adjusted to 4.0 before autoclaving. The segments were grown in 125 cc Erlenmeyer flasks with 50 cc of medium and 3 stem pieces per flask and were kept in cupboards in a hallway of the building with temperature fairly constant at 25°C. They received weak diffuse illumination from the electric ceiling lights. In the first experiment, analyses were made after 65 days; in the second, samples were taken at the start and at intervals during a 52 day period. Analyses for reducing sugar and sucrose were carried out by the method of Forsee(8) and starch was extracted and converted to reducing sugar by the method of Pucher, Leavenworth and Vickery(9).

Results. The results obtained in the first experiment are presented in Fig. 1. It may be seen from curve 1 that the average dry weight per piece increased with the concentration of I.A.A. up to 1 mg/l and was somewhat lower for the 10 mg/l treatment. The values for fresh weights (curve 2), which may be more indicative of total growth, are plotted on one-tenth the scale and correspond closely with the values for the dry weights. The concentrations of sucrose plus reducing sugar (curve 3), reducing sugar (curve 4), starch (curve 5), and sucrose (curve 6) are all plotted as per cent of dry weight. The sucrose concentration of the tissues (curve 6)

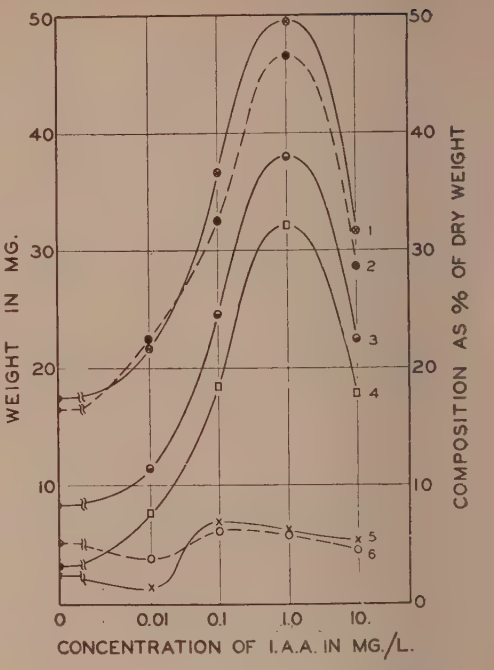

FIG. 1.

Effects of I.A.A. concentrations on growth and changes in carbohydrate concentrations of tobacco stem segments cultured *in vitro*. Experiment started 11/14/49, harvested 1/18/50. Curve 1, average dry weight; curve 2, average fresh weight × 1/10 in mg/segment. Curves 3, sucrose + reducing sugar; 4, reducing sugar; 5, starch, and 6, sucrose concentrations of segments expressed as % of dry weight. All data are values from 27 to 42 segments.

was practically unaffected by the I.A.A. concentration and remained at approximately 5%, which is about 0.5% of the fresh weight or only one-fourth of the sucrose concentration of the external medium at the beginning of the experiment. On the other hand, the reducing sugar concentration showed a striking increase with increasing concentrations of I.A.A. up to 1 mg/l and then decreased, so that the curve for the reducing sugar concentration in the tissues closely parallels the curve for the weights of the stem segments. The starch concentrations were much lower than those of sugar. In this experiment the starch concentrations increased somewhat with the I.A.A. treatments, but the correlation with weights of pieces and concentrations of I.A.A. is less definite than

7. Skoog, F., and Tsui, C., *Am. J. Botany*, 1948, v35, 782.

8. Forsee, W. T., *Ind. Eng. Chem., An. Ed.*, 1938, v10, 411.

9. Pucher, G. W., Leavenworth, C. S., and Vickery, H. B., *Anal. Chem.*, 1948, v20, 850.

TABLE I. Effects of IAA on Weights, Sugar, and Starch Concentrations in Tobacco Stem Segments Grown *in Vitro*.
Experiment started 2/10/50.

Age of cultures (days)	Treatment	No. of pieces per sample	Fresh wt		Dry wt		Reducing sugar		Sucrose		Starch	
			Avg per piece (mg)	Diff. from control	Avg per piece (mg)	Diff. from control	% of dry matter	Diff. from control	% of dry matter	Diff. from control	% of dry matter	Diff. from control
0		48	77		5.7		8.1		9.4		4.1	
21	Control	15	134		10.0		7.2		9.0		1.9	
	Low IAA	26	132	—2	10.0	0.0	7.6	+0.4	11.9	+2.9	2.1	+0.2
	High IAA	21	206	+72	13.0	+3.0	13.8	+6.6	13.0	+4.0	0.6	—1.3
36	Control	15	104		8.2		5.6		11.9		3.7	
	Low IAA	23	138	+34	11.1	+2.9	7.8	+2.2	8.9	—3.0	1.9	—1.8
	High IAA	18	263	+159	16.7	+8.5	21.7	+16.1	14.0	+2.1	1.0	—2.7
52	Control	15	163		13.6		5.3		10.1		(lost)	
	Low IAA	26	129	—34	11.1	—2.5	6.5	+1.2	10.8	+0.7	3.3	
	High IAA	21	339	+176	24.0	+10.4	23.7	+18.4	15.5	+5.4	2.2	

Low IAA = Mean for treatments with 0.01 and 0.1 mg/l of IAA. High IAA = Mean for treatments with 1.0 and 10.0 mg/l of IAA.

for reducing sugars. Since the values are low, the discrepancies may be within the experimental error. These curves demonstrate a definite correlation between the applied auxin concentrations, the extent of growth of the tissues, and their reducing sugar concentrations. Furthermore, since the tissues with most growth had the highest concentrations of both sugars and starch, the high reducing sugar content cannot have been derived from storage products but must have been formed by the tissues in response to the treatments with I.A.A.

In a second experiment samples were analyzed at successive stages of growth. The data obtained at the start and after 21, 36, and 52 days of growth are shown in Table I. In this experiment there was no significant difference in growth between cultures with 0.01 and 0.1 mg/l of I.A.A. added to the medium or between those with 1.0 and 10 mg/l of I.A.A.; therefore mean values for the former two treatments are presented in the table as "low I.A.A." and mean values for the latter two treatments are presented as "high I.A.A."

The data for fresh and dry weights indicate that control pieces and those supplied with low I.A.A. concentrations increased in weight to about the same extent, but much less than did the pieces supplied with the high I.A.A. concentrations. The reducing sugar content was 8.1% at the start and decreased to about 5.5% in the controls during the growth period. With the low I.A.A. treatments the mean decrease in reducing sugar was less than in controls, while with the high I.A.A. treatment the reducing sugar markedly increased with time to a mean value of 24% of the total dry weight. Comparisons of the data for weights of pieces and their reducing sugar concentrations again show a close agreement between increase in size and reducing sugar concentration. Furthermore, it may be stressed that the values for reducing sugar obtained after long periods show net losses in control pieces, which grew but little, as against marked increases in tissues supplied with high I.A.A. concentrations, which grew rapidly. The sucrose con-

centrations of the tissues with low I.A.A. treatments and of controls did not change appreciably during the growth period, but showed a definite, small increase with high I.A.A. treatments. The starch concentrations of the tissues were again relatively low, and in contrast with the results after 65 days growth in the first experiment, there was no increase from treatments with I.A.A.

Discussion and conclusions. The present results demonstrate a definite correlation between the effects of different concentrations of I.A.A. on growth and on reducing sugar concentrations in the tissues. To our knowledge this is the first case of such a relationship between a metabolic constituent and growth of tissues in response to treatments with an auxin.

We have no explanation for the contrast between these results and reports in the literature other than that differences in methods and plant materials may be responsible. The tobacco tissues we used have a low endogenous auxin content and they were analyzed after long growth periods with added I.A.A.; however definite increases in reducing sugar from treatments with I.A.A. may be obtained within the first week. Furthermore, the tissues were growing continuously and particularly those treated with I.A.A. continued to increase both in size and dry weight, whereas earlier tests have been performed on tissues capable of only limited growth in length and undergoing marked losses in dry weight regardless of the treatments. It should be pointed out that in the present work a correlation is demonstrated between increase in weight, rather than growth rate of the pieces, and the concentration of reducing sugar. This distinction may not be important but will be investigated. The reducing material has been expressed in terms of glucose but has not yet been characterized, and may not be simply glucose.

Conclusion. Auxin has a catalytic function in carbohydrate metabolism which is related to its effect on growth but which is not primarily concerned in the respiratory degradation of sugars. The above results are, therefore, reported at this time as they may be of general interest in studies of the mechanism of auxin action in the growth of plants.

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Effect of Carbohydrate on Growth Response to Vitamin B_{12} in the Hyperthyroid Rat.* (17974)

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A rat assay for the estimation of vitamin B_{12} in biological materials previously reported (1) makes use of a corn-soybean meal basal ration containing iodinated casein. Ershoff (2), however, has noted that no increase in growth is obtained with vitamin B_{12} in rats fed thyroid active materials when a purified sucrose-casein diet is used. Whole liver, on the other hand, gives a maximum response. Betheil and Lardy(3) report that vitamin B_{12} will partially overcome the growth retardation in the hyperthyroid rat on the sucrose-casein ration. In his latest report Ershoff(4) demonstrates that full-fat soybean

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by a grant from the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation.

1. Register, U. D., Ruegamer, W. R., and Elvehjem, C. A., *J. Biol. Chem.*, 1949, v177, 129.

2. Ershoff, B. H., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 209.

3. Betheil, J. J., and Lardy, H. A., *J. Nutrition*, 1949, v37, 495.

4. Ershoff, B. H., *J. Nutrition*, 1949, v39, 259.

TABLE I.
Comparison of Growth Response to Vitamin B_{12} in Rats on the Corn-Soybean Meal and Sucrose-Casein Rations.

Ration	Avg growth 3-wk period, g	Food consumption, g/rat/day
Corn-soybean basal (0.1% iodinated casein)	52	13.2
" " " " " " + 0.2 μg B_{12}/day	110	—
Sucrose-casein basal (0.1% iodinated casein)	52	—
" " " " " " + 0.2 μg B_{12}/day	69	—
Corn-soybean basal (0.1% iodinated casein) + 10% corn oil	61	10.4

meal has an antithyrotoxic effect and he correlates its effectiveness with the amount of fat present in the meal. Furthermore, with his sucrose-casein regime, soybean oil, as well as several other animal and vegetable fats, was capable of significantly ameliorating the decreased rate of growth in the hyperthyroid rat. The following work was undertaken to further elucidate the nature of these observed antithyrotoxic effects as they may be related to the specificity of the rat assay for vitamin B_{12}.

Experimental. Two basal rations were used. The corn-soybean basal diet was identical to that employed in the rat assay for vitamin B_{12} except that the level of most of the vitamins was doubled(1). Its composition is as follows: corn meal (5% fat) 45%, commercial soybean meal (5% fat) 45%, salt mixture† 2%, corn oil 5%, cystine 0.3%. The vitamins were added in the following amounts: choline chloride 100, thiamine hydrochloride 0.6, pyridoxine hydrochloride 0.4, calcium pantothenate 4.0, niacin 4.0, folic acid 0.05, biotin 0.02, p-amino-benzoic acid 25, riboflavin 0.6 and inositol 20 mg/100 g ration. In addition each rat received 2 drops of halibut liver oil weekly. In the sucrose-casein ration, sucrose 63% and casein‡ 18%, replaced the corn and soybean meals in the above diet but otherwise it was identical. Crystalline vitamin B_{12} when used was administered by injection. The desiccated thyroid§ and iodinated casein‖ were mixed directly into the rations. Male weanling rats (Sprague-Dawley strain), within a weight range of 40 to 45 g at the start of an experiment, were employed. The average gain in weight during the first three weeks of growth was used as a measure of activity. Individual cages were used with feed and water being given *ad libitum*.

Results and discussion. Comparison of the growth promoting activity of vitamin B_{12} in rats on the sucrose-casein and the corn-soybean meal rations containing 0.1% iodinated casein is shown in Table I. On the corn-soybean diet, growth was approximately doubled over a 3-week period with vitamin B_{12} supplementation. Only slight improvement was noted upon addition of vitamin B_{12} to the sucrose-casein ration.

This partial growth stimulation to vitamin B_{12} with the diet containing sucrose and casein is in agreement with the results of Betheil and Lardy(3). A possible explanation for the difference in these data and those of Ershoff may lie in the nature of the rations used. The rats in the experiments being reported, and likewise those used by Betheil

† Salts IV. *J. Biol. Chem.*, 1935, v104, 657.

‡ Vitamin test casein, General Biochemicals, Inc., Chagrin Falls, O.

§ Thyroid powder, U.S.P., Wilson Laboratories, Chicago, Ill.

‖ Supplied by Dr. W. R. Graham, Cerophyl Laboratories, Inc., Kansas City, Mo.

TABLE II.
Antithyrotoxic Effect of Soybean and Corn Meals in Sucrose Casein Ration Containing 0.5% Desiccated Thyroid.

Ration	Avg growth 3 wk period, g
Sucrose-casein basal	58
" " " + 10% whole liver powder	88
" " " + 1 μg B_{12}/day	66
" " " + 45% corn meal + 1 μg B_{12}/day	94
" " " + 45% soybean meal + 1 μg B_{12}/day	83

and Lardy, received 5% corn oil in their diet, while Ershoff employed a fat free basal ration and supplemented his animals with 500 mg cottonseed oil 3 times a week. This is significant in the light of Ershoff's report (4) that corn oil, as well as other animal and vegetable fats, exerts an antithyrotoxic effect. It was thought that the nature of this effect might possibly be attributed to food consumption. Two groups were fed the corn-soybean ration (0.1% iodinated casein) while one received an additional 10% corn oil. The growth and food consumption data are shown in Table I. The animals fed the increased amount of corn oil grew slightly better than those on the regular corn-soybean basal. Their food consumption, however, was less (20%). Therefore, by consuming less feed, less iodinated casein was ingested which could account for the growth response.

The possibility that corn meal might possess the antithyrotoxic activity found with soybean meal was next investigated. Corn meal and soybean meal were added individually at a 45% level to the sucrose-casein basal ration at the expense of the carbohydrate and protein of this diet. The total amount of protein was kept constant in the different rations. 0.5% desiccated thyroid and 1 μg per day of injected vitamin B_{12} were used. As shown in Table II, a response to vitamin B_{12} was observed when both the corn and soybean flours were used. The amount of corn meal needed to produce the stimulatory effect was then determined. From the data in Table III it is seen that at least 30% corn meal must be included in the diet to obtain maximum growth promotion when 0.3% iodinated casein is used. This amount of iodinated casein is approximately equivalent to 0.5% desiccated thyroid.

TABLE III.
Growth Response to Vitamin B_{12} of Rats on Casein-Sucrose Ration Containing 0.3% Iodinated-Casein Supplemented with Graded Levels of Corn Meal.

Supplement	Avg growth 3 wk period, g
None	48
0.2 μg B_{12}/day (inj.)	51
" " " + 5% corn meal	52
" " " + 10% " "	60
" " " + 20% " "	60
" " " + 30% " "	69
" " " + 45% " "	69

The similar effectiveness of corn meal and soybean meal suggested that the nature of the carbohydrate in a diet may determine its antithyrotoxic activity. The validity of this idea was borne out by the following experiment. Dextrin and corn starch were substituted for the sucrose in the sucrose-casein ration. The data are presented in Table IV. The addition of either dextrin or corn starch counteracted the growth depression noted when sucrose served as the carbohydrate. Vitamin B_{12} produced a significant growth response when administered to the dextrin and corn starch regimes but only a slight increase in conjunction with the sucrose ration.

Defatted corn meal (Soxhlet extraction) when tested in the sucrose-casein basal ration at a level of 45% possessed activity equal to that of the dextrin and corn starch. The crude fat that was extracted produced some growth stimulation when included at a 2% level. These results are presented in Table IV.

The manner in which the carbohydrate of the diet functions to produce the growth stimulation noticed is not readily explained.

TABLE IV.
Effect of Carbohydrate in Producing a Growth Response to Vitamin B_{12}.

Ration	Avg 3 week growth, g
Sucrose-casein basal + 0.15% iodinated casein	60
" " " + " " " + 0.5 μg B_{12}/day	69
" " " + 0.25% " "	54
" " " + " " " + 0.5 μg B_{12}/day	65
Dextrin-casein basal + 0.15% " "	66
" " " + " " " + 0.5 μg B_{12}/day	93
" " " + 0.25% " "	76
" " " + " " " + 0.5 μg B_{12}/day	90
Corn starch-casein basal + 0.25% iodinated casein	77
" " " + " " " + 0.5 μg B_{12}/day	87
Sucrose-casein basal + 45% defatted corn meal + 0.25% iodinated casein + 0.5 μg B_{12}/day	85
Sucrose-casein basal + 2% extracted corn fat + 0.25% iodinated casein + 0.5 μg B_{12}/day	73

Ershoff(2) has reported an antithyrotoxic factor present in extracted liver residue; however, the exact nature of this substance or its origin is not known. The observed phenomenon with liver powder might possibly be attributed in part to the liver glycogen since activity was reported only with the residue fed at a 10% level. Altering the carbohydrate of the ration may function in producing a more favorable synthesis within the intestinal tract of other required factors.

Summary. The failure of rats to respond completely to vit. B_{12} on a sucrose-casein diet containing a thyroid active material can be overcome by substituting defatted corn meal, corn starch or dextrin for the sucrose. The nature of this effect may possibly be attributed to the intestinal synthesis of other required factors. An explanation for the counteractive effect of corn oil on the growth depression in the hyperthyroid rat is offered.

We are indebted to Merck and Co., Inc., Rahway, N. J., for crystalline vitamins including B_{12}, and to Dr. B. L. Hutchings of the Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y., for synthetic folic acid.

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Role of Protogen in the Nutrition of an Unidentified Corynebacterium (17975)

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The protozoon *Tetrahymena geleii* has been shown by Dewey(1) to require an unknown growth factor. Some of the properties of this factor were described by Stokstad *et al.*(2) and the name "protogen" was suggested. Kidder and Dewey(3) have shown that *T. geleii* will grow rapidly on a chemically defined medium with the addition of relatively small amounts of concentrates of protogen. We wish to report the findings of a bacterium which also requires protogen for growth.

Experimental and results. The organism* is a gram positive bacillus, 0.2 by 1.0μ, isolated from a sample of cow manure. It grows under aerobic conditions on a wide variety

1. Dewey, V., *Biol. Bull.*, 1944, v87, 107.
2. Stokstad, E. L. R., Hoffman, C. E., Regan, M., Fordham, D., and Jukes, T. H., *Arch. Biochem.*, 1949, v20, 75.
3. Kidder, G. W., and Dewey, V., *Arch. Biochem.*, 1949, v20, 433.

* We wish to thank Dr. M. A. Petty of Lederle Laboratories for the culture of this organism.

TABLE I.
Composition of Medium.

	Amt per liter of final medium
Acid hydrolyzed casein	5 g
Dextrose	5 "
Sodium acetate (anhydrous)	2.5 "
Speakman salt solution A	5 ml
" " " B	5 "
Asparagine	50 mg
DL-tryptophane	100 "
L-cystine	100 "
Adenine	10 "
Guanine	10 "
Uracil	10 "
i-inositol	5 "
Choline chloride	5 "
Calcium pantothenate	.5 "
Thiamine	.5 "
Nicotinamide	.5 "
Riboflavin	.5 "
Pyridoxine	.5 "
P-aminobenzoic acid	.5 "
Pteroylglutamic acid	.5 "

of carbohydrates. These carbohydrates include arabinose, galactose, glucose, mannose, rhamnose, xylose, lactose, maltose, sucrose, trehalose, dextrin, inulin, raffinose, starch, dulcitol, glycerol, mannitol, sorbitol, and inositol. It is non-hemolytic, does not liquefy gelatin, does not produce nitrites and nitrates and does not produce ammonia from urea. It gives a positive catalase test. The morphology, growth and staining characteristics indicate that it is closely related to *Corynebacterium bovis*.†

The culture was maintained on a yeast extract glucose agar. An inoculum was prepared by growing the organism at 25°C for 48 hours on the basal medium (Table I) plus refined liver extract. The cells were centrifuged, resuspended in an equal volume of saline and one drop used per 2 ml assay tube. The incubation temperature was 25°C. The organism was incapable of growing on the basal medium (Table I) unless natural supplements such as refined liver extract or yeast were added. It was then found that growth could be produced by a concentrate of protogen containing 20,000 units‡ per milligram of solids. The results (Table II) show that 0.02 unit of protogen, contained in 0.001 μg of solids, produced half maximum growth after 40 hours of incubation and almost maximum growth after 88 hours. It appeared that protogen was the only growth factor required as maximum growth was obtained with the protogen concentrate when a 40 or 88 hour incubation period was used.

Discussion. Since the completion of these experiments, Snell and Broquist(4) have suggested the identity of protogen with the "acetate factor" of Guirard *et al.*(5) These workers had previously demonstrated that small amounts of acetic acid function as a growth stimulant for lactic acid bacteria. Concentrates of natural materials are capable of replacing acetic acid and are more active per unit of weight than acetic acid itself. In the experiments of Snell and Broquist, concentrates of protogen were active for *Lactobacillus casei* as sources of the "acetate factor." This suggests a relationship between protogen and the metabolism of acetic acid in lactic acid organisms. While *L. casei* requires protogen only in the absence of acetate, both *T. geleii* and the corynebacterium described here need protogen even in the presence of sodium acetate. It has been reported, however, by Kidder and Dewey(6) that the

TABLE II.
Effect of Protogen on Growth of an Unidentified Corynebacterium.

Supplement per 2 ml medium	Growth (optical density) 40 hr	88 hr
None	.025	.07
.01 μl refined liver extract	.25	.47
.03 " " " "	.35	.76
.10 " " " "	.41	.90
1.0 " " " "	.67	1.25
.02 unit protogen*	.35	.77
.06 " "	.45	.95
.20 " "	.55	1.02
.60 " "	.65	1.05

* Supplied as a concentrate containing 20 units per μg of solids.

† We are indebted to Dr. A. Zink of Lederle Laboratories for classifying the organism and determining its morphological and cultural characteristics.

‡ One unit of protogen is the activity contained in one mg of a standard liver preparation.

4. Snell, E. E., and Broquist, H. P., *Arch. Biochem.*, 1949, v23, 326.

5. Guirard, B. M., Snell, E. E., and Williams, R. J., *Arch. Biochem.*, 1946, v9, 381.

growth rate of *T. geleii* can be increased by the addition of 1.0 mg per ml of sodium acetate.

The requirement of the different organisms for protogen differs widely. The requirements of *T. geleii*, the corynebacterium, and *L. casei* for protogen are respectively 0.5, 0.01 and 0.025 unit per ml for maximum growth. The protogen requirements for *L. casei* can be replaced by 0.25 mg of sodium acetate per ml.

Summary. An unidentified gram-positive bacillus has been found which requires protogen for growth in about one-fiftieth of the concentration required by *Tetrahymena geleii.*

6. Kidder, G. W., and Dewey, V., *Arch. Biochem.*, 1949, v20, 433.

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Applicability of a Differential Analyzer to Determination of Protein Fractions by the Electrophoretic Technic. (17976)

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This laboratory has found it advantageous to use a differential analyzer in place of a planimeter in connection with determination of concentrations of plasma protein fractions from electrophoretic patterns. The differential analyzer is an analogue computer developed at the Massachusetts Institute of Technology under the direction of Dr. Vannevar Bush. One was recently acquired by Wayne University. Its use was made available to us and the work was carried out at Wayne University Computation Laboratory under the direction of Prof. Arvid W. Jacobson.

Although the primary purpose of the analyzer is the solution of differential equations(1) the principles upon which it operates are quite simple. All quantities involved in the calculations are represented by the rotation of shafts. The fundamental unit is the integrator which is shown in Fig. 1. The disc A turns the wheel B by friction. Hence, a slight amount of rotation, $\triangle W$ of wheel B will be proportional to the product of the corresponding rotation, $\triangle V$ of disc A and the distance U of wheel B from the center of A; *i.e.*,

$$W = k \cdot U \triangle V$$

Thus, if u and v vary, the rotation of wheel B will be represented by the integral

$$W = k \int U dV.$$

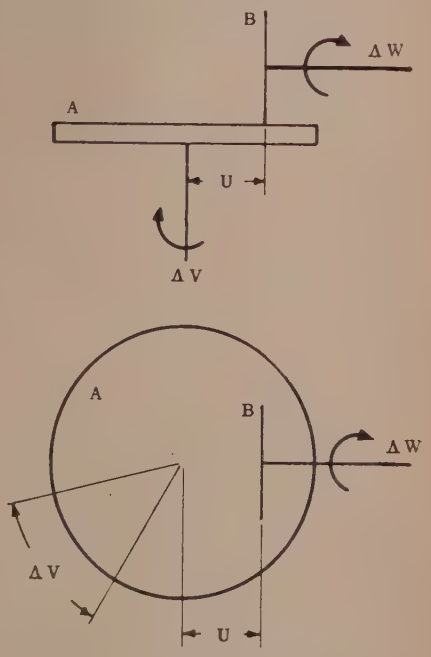

FIG. 1.
Integrator.

Fig. 2 shows a schematic diagram of the entire operation. In this drawing the mecha-

1. For a complete discussion of the differential analyzer see Bush, V., and Caldwell, S. H., *J. Franklin Inst.*, 1945, v240, 255.

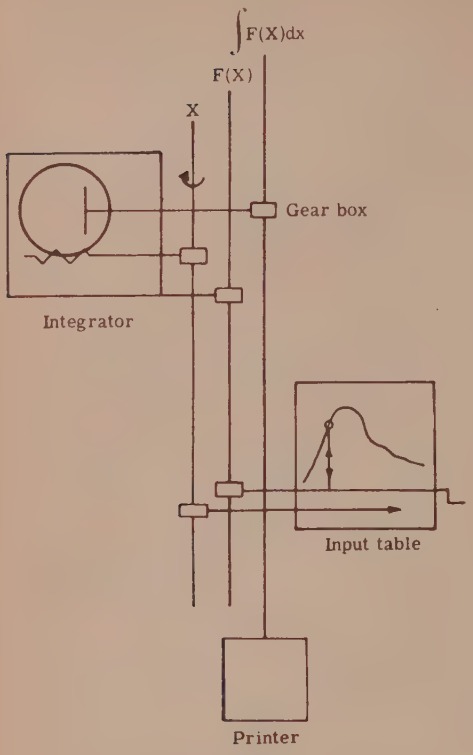

FIG. 2.
Schematic drawing of entire operation.

nism of Fig. 1 is represented by the symbol marked integrator. The symbol marked input table is a function unit used to generate the function F(X) in the machine. A graph of this function is plotted on the table, and the machine moves the carriage, to which an index or bull's-eye is attached, horizontally across the input table, while the operator works the crank in such a way as to keep the bull's-eye on the curve. The speed of the horizontal motion of the bull's-eye can be varied by the operator, depending on the slope of the curve to be followed. This arrangement, among other things, accounts to a large extent for the high accuracy of the machine. While the planimeter operator has to follow the curve along both dimensions of the plane with the point of a needle, the operator of the differential analyzer need concern himself with the vertical adjustment only. Moreover, studies at M.I.T. have shown that the human eye can make optimal decisions in following the curve by means of the circle of a bull's-eye, whose area is bisected by the curve.

The operation of the machine begins with application of power to rotate the shaft designated X. This shaft is connected to a cross shaft which moves the bull's-eye across the input table where the operator generates the function F(X) by means of the crank. The integrator integrates F(X) with respect to X, and this quantity is represented by the rotation of the top shaft, which is directly connected to the printer. The printer has a capacity of 5 digits and may be actuated by the operator at any desired moment.

The tracing of a typical ascending electrophoretic pattern is shown in Fig. 3. The areas to be evaluated in order from left to right represent concentrations of the following plasma protein fractions: albumin, alpha$_1$, alpha$_2$ and beta globulins, fibrinogen, gamma globulin and the delta anomaly.

Table I shows a comparison of the results obtained by measuring the areas of three tracings 5 times on the differential analyzer, and 5 times by means of a precision disc planimeter(2). The results clearly show that the differential analyzer measurements have

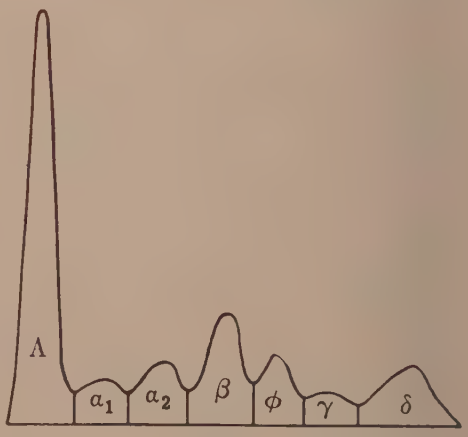

FIG. 3.
Typical electrophoretic pattern.

2. G. Coradi, Zurich, Switzerland.

TABLE I.
Means and Standard Deviations for 5 Measurements of 3 Tracings by 2 Methods.

	Differential analyzer		Disc planimeter	
	Mean Sq. in.	Stand. dev.* Sq. in.	Mean Sq. in.	Stand. dev. Sq. in.
	Tracing No. 1			
Albumin	1.302	.002	1.288	.048
$Alpha_1$	.106	.005	.113	.014
$Alpha_2$	.228	.008	.227	.005
Beta	.310	.005	.313	.007
Phi	.204	.004	.202	.005
Gamma	.407	.009	.401	.008
Delta	.362	.014	.361	.007
Total area	2.918	.045	2.905	.066
	Tracing No. 2			
Albumin	1.244	.006	1.237	.010
$Alpha_1$	.162	.005	.168	.006
$Alpha_2$	.291	.002	.295	.008
Beta	.386	.002	.384	.010
Phi	.223	.002	.223	.002
Gamma	.480	.005	.484	.006
Delta	.406	.007	.404	.008
Total area	3.190	.028	3.194	.028
	Tracing No. 3			
Albumin	1.576	.006	1.526	.052
$Alpha_1$	.179	.004	.180	.008
$Alpha_2$	.243	.003	.248	.007
Beta	.393	.002	.395	.005
Phi	.160	.003	.163	.010
Gamma	.298	.006	.313	.016
Delta	.398	.005	.408	.009
Total area	3.248	.020	3.233	.062

*Standard deviation $S = \sqrt{\frac{\Sigma(x_i - \bar{x})^2}{n-1}}$.

greater precision than those obtained with the planimeter, and that the greatest actual differences arise in measurements of the largest areas, namely those of the albumin peak, and of the total area of the pattern. Considering percentage deviations from the mean, the maximum deviation for the set of 120 measurements on the differential analyzer was 7.3%, with 88% of the deviations below 2%, and 31% below 0.5%. The same set of measurements by means of the planimeter yielded a maximum deviation from the mean of 22.1%, with 61% of the values below 2.0% and 21% below 0.5%.

In addition to obtaining more accurate results, use of the differential analyzer will save a great deal of time, especially when comparatively large numbers of tracings can be run at one time. While it takes approximately 2 hours to set up the mechanism for the operation, the time required for measurement of a tracing is only 10 minutes. Use of the planimeter requires approximately 1 hour per tracing, since the lack of precision made it necessary to measure each tracing at least twice.

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Simultaneous Bilateral Determinations of Cerebral Blood Flow and Arterial-Cerebral Venous Oxygen and Glucose Differences.* (17977)

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The validity of the nitrous oxide procedure for measuring cerebral blood flow depends upon the assumption that blood obtained from one internal jugular bulb is representative of mixed cerebral venous blood. Since the brain is actually a heterogeneous organ, comprised of a wide variety of cell types, and with different rates of metabolism in its various parts, the nitrous oxide technic at best yields an average value for the blood flow through and the metabolism of these different areas. Unless the blood draining these parts is reasonably well mixed, or at least consistently distributed, it is manifestly impossible to reach valid conclusions concerning

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either local or whole brain metabolism by sampling blood from one internal jugular bulb. These studies were undertaken because a difference of opinion concerning the mixing and distribution of cerebral venous blood is apparent in the literature(1-5) and as a preliminary to further observations on the circulatory dynamics of the brain. The nitrous oxide technic, as devised by Kety and Schmidt(1) and as modified in this laboratory(6) was used to measure cerebral blood flow and metabolism in 18 simultaneous bilateral observations in 12 subjects.

Method. The subjects chosen represented several disease states, since it was felt that the validity of the method could be tested better in this way than with normal subjects alone. Three of the patients (A.C., A.P., and J.S., Table I) had clinical evidence of unilateral cerebral disease. The nitrous oxide technic has been described elsewhere (1); the modification in use in this laboratory consists of drawing simultaneous, integrated samples from artery and vein throughout the 10 minute period of nitrous oxide inhalation(6). In every instance samples taken for blood flow determination and oxygen and glucose contents were drawn simultaneously from the artery and right and left internal jugular bulbs. Blood oxygen content was measured by the spectrophotometric method of Hickam and Frayser(7); blood glucose content was measured by Nelson's modification of Somogyi's method(8). Samples for blood oxygen content were drawn before and after each blood flow determination so that each arterial-cerebral oxygen difference is an average of two determinations. Two samples for blood glucose content were drawn from the artery and both jugular bulbs before and after each blood flow determination so that each arteriovenous glucose difference is an average of 4 determinations. Mean arterial pressures used to calculate cerebral vascular resistance were measured by the auscultatory method.

Results. The data are presented in detail in Table I. The p values were calculated by comparing the differences between the right and left determinations in the individual subjects. It is apparent that there is no significant difference between the two sides in any of the measured cerebral metabolic values. The deviations are all within the experimental error of these determinations as performed in this laboratory. The 3 patients with unilateral cerebral disease did not differ from the remainder of the subjects in this respect.

Comment. The prevailing disagreement concerning the percentage of subjects in whom longitudinal and straight sinus blood mix completely in the torcular(9-12) does not particularly bear on the problem of the validity of the nitrous oxide procedure. It is agreed that if this were the only mechanism whereby blood from the various parts of the brain were mixed, the nitrous oxide technic would be invalid in a considerable percentage of cases. Anatomical studies reported by Shenkin, Harmel, and Kety(13) indicate adequate opportunity for cortical-subcortical mixing of blood draining from the various histologic areas of the brain. The studies reported here support those observations and indicate that regardless of the anatomical variations in different individuals, the calculated cerebral blood flows and oxygen and glucose contents of simultaneously drawn

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TABLE I.

Bilateral, Simultaneously Determined, Cerebral Metabolic Values.

Patient	Age	Remarks	Cerebral blood flow, ml/min./100 g brain		Arterial-cerebral venous O_2 diff. vol. %		Arterial-cerebral ven. glu. diff. mg %		Cerebral oxygen consump. ml O_2/min. 100 g brain		Cerebral glucose consump. mg glucose/min./100 g brain		Cerebral vascular resist.	
			R.	L.	R.	L.	R.	L.	R.	L.	R.	L.	R.	L.
E.	47	Cerebral vascular dis.												
		Before stellate block	51	50	6.4	6.5	—	—	3.2	3.2	—	—	2.84	2.90
		After stellate block (rt.)	48	47	6.8	7.1	10	13	3.2	3.3	4.8	6.1	2.96	3.02
O.	63	Cerebral vascular dis.												
		Before stellate block	44	45	5.2	5.7	—	—	2.3	2.6	—	—	2.50	2.45
		After stellate block (rt.)	37	32	6.7	6.9	9	7	2.5	2.2	3.3	2.2	2.78	3.22
C.P.	35	Subarachnoid hemorrhage	40	35	6.3	7.1	—	—	2.5	2.5	—	—	2.20	2.51
A.C.	37	Mitral stenosis. Embolism to cortical branch of rt. middle cerebral artery	32	35	7.7	7.7	8	8	2.5	2.7	2.6	2.8	3.02	2.77
A.P.	57	Embolism to branch of left middle cerebral artery before stellate block	—	—	5.4	5.3	10	12	—	—	—	—	—	—
		After stellate block (left)	—	—	5.9	5.2	11	11	—	—	—	—	—	—
C.S.	29	Coarctation of aorta	43	45	8.0	7.3	8	8	3.4	3.3	3.4	3.6	3.05	3.19
J.W.	42	Rheumatoid arthritis serial detm's. (1)	39	41	—	—	11	13	—	—	4.3	5.3	2.05	1.95
		(2)	50	51	—	—	12	14	—	—	6.0	7.1	—	—
		(3)	43	47	—	—	12	11	—	—	5.2	5.2	—	—
J.S.	37	Berry aneurysm, left int. carotid artery. Before ligation	71	73	4.4	4.4	10	11	3.1	3.2	7.0	7.8	1.39	1.36
		After ligation of left int. carotid artery	—	—	4.9	4.9	7	5	—	—	—			—
R.	36	Hysteria. Serial detm's (1)	66	57	5.1	5.6	12	12	3.3	3.2	7.9	6.8	—	—
		(2)	57	52	5.3	6.3	13	12	3.1	3.3	7.4	6.2	—	—
		(3)	56	51	6.9	6.7	10	9	3.8	3.4	5.6	4.6	—	—
W.	30	Tic doloreux, before stellate block	75	70	6.5	6.6	13	14	4.9	4.8	9.7	9.7	1.27	1.36
		After stellate block (left)	60	53	6.3	6.3	11	12	3.8	3.4	6.6	6.4	1.59	1.79
S.	68	Simple depression	39	41	7.0	7.0	12	11	2.7	2.9	4.9	4.7	2.30	2.20
E.	22	Myasthenia gravis	77	69	5.6	5.2	9	8	4.3	3.6	6.7	5.5	1.20	1.30
		Mean	51.6	49.7	6.13	6.21	10.4	10.6	3.24	3.17	5.69	5.60	2.24	2.31
		p values	>0.05		>0.3		>0.6		>0.3		>0.5		>0.2	

$t = \frac{\text{Mean diff.}}{\text{Std. error of mean diff.}}$.

R = Right. L = Left.

samples from the right and left internal jugular bulbs do not differ appreciably on the two sides in subjects at rest. These data also suggest that adequate mixing of blood from the two cerebral hemispheres may occur in the presence of unilateral cerebral disease. They are not in agreement with the findings reported by Ferris and his colleagues(2); this discrepancy remains unresolved. These data would also cast doubt on the assumption that accurate differential values for metabolism of the cortex and lower portions of the brain can be adduced from bilateral internal jugular bulb samples(5,14).

Summary and conclusions. 1. The values for cerebral blood flows, arterial-cerebral venous oxygen and glucose differences were not significantly different on the two sides in 18 simultaneous bilateral observations in 12 subjects. 2. Three subjects with unilateral cerebral disease also had similar values on the two sides. 3. The assumption that blood obtained from one internal jugular bulb is representative of mixed cerebral venous blood appears to be correct.

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Diurnal Variations of Renal Function in Congestive Heart Failure. (17978)

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Employing a constant intravenous infusion technic, Sirota, Baldwin and Villarreal(1) have examined the diurnal variations of renal function in normal subjects. These studies reveal a significant decrease in urine flow during sleep, resulting primarily from increased tubular reabsorption of water, with only a slight and questionably significant decrease in glomerular filtration rate from 12 midnight to 4 a.m. The renal plasma flow did not vary significantly throughout the 24 hour period. The present report is concerned with similar studies in patients with congestive heart failure.

Methods. Simultaneous endogenous creatinine chromogen, inulin and p-aminohippurate clearances were determined during 4 to 6 hour periods throughout 24 hours in 10 male subjects in congestive heart failure. In 6 subjects sodium excretion was also followed. In 5 subjects cardiac failure was associated with rheumatic heart disease, in 4 with arteriosclerosis, and in 1 with cor pulmonale. Six of these patients at the time of study had persistent or increasing peripheral edema in spite of complete bed rest, low salt diet and complete digitalization. Of the other 4, 2 were edema-free and 2 were undergoing diuresis, spontaneous in 1 case and in the other as a result of digitalization. With the exception of 1 individual (E.), mercurial diuretics were withheld for a minimum of 5 days prior to study. The test substances were administered by means of a constant delivery pump at an approximate rate of 0.3 cc/min. Five per cent glucose in distilled water was used as the diluent.§ Analytical methods and details of technic in continuous 24 hour clearance studies have been reported previously(1).

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‡ Aided by a grant from the Commonwealth Fund.

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§ PAH dissolved in 5% glucose solution forms a reaction production in which the p-amino group is covered. This product apparently has a low clearance, as indicated in the low extraction ratios of PAH-glucose mixtures(2). For this reason all data on C_{PAH} are deleted from the report.

Urine sodium was determined with the Perkin-Elmer internal standard flame photometer.

Results. Data on day (D) and night (N) values of urine flow, inulin U/P ratio, inulin clearance (C_{IN}), endogenous 'creatinine' chromogen clearance ($C'_{CR'}$) and sodium excretion are recorded in Table I. The night value in each case represents the mean value obtained for the periods corresponding roughly to the sleeping hours for that individual; the day values are the means of all other observations.

Discussion. While the number of patients studied in this series is small, certain conclusions appear to be warranted. In the group with persistent edema the urine flow either remained constant or increased, in contradistinction to the decrease in urine flow which occurs during sleep in normal subjects. In all the edematous subjects, the filtration rate increased to some extent (6 to 35%) during sleep, whereas in normal subjects this function remains unchanged or decreases(3). In normal subjects the nocturnal decrease in urine flow is almost wholly attributable to increased reabsorption of water. The data suggest that the increase in filtration rate may be causally related to increased urine flow in three subjects (J.Me., F. and G.), but the problem does not permit accurate analysis at this time. This reversal of the diurnal variation in urine flow and filtration rate pattern in patients with persistent edema does not appear to be directly related to the level of glomerular filtration since it occurred in J.Me. with a day-time filtration rate of 92 cc/min and in F. with a day-time filtration rate of 47.9 cc/min.

The reversal of diurnal pattern was not observed in 2 patients with left ventricular heart failure but without peripheral edema (J.T. and A.D.), or in 2 patients undergoing diuresis spontaneously (J. Ma.) or in consequence of digitalization (T.P.).

Chloride(4-7) and sodium(7-8) excretion typically decrease in normal subjects during sleep. In contrast, 2 of our 3 subjects with edema showed a marked increase in sodium excretion at night (N/D ratio of 1.54), and the third showed a slight increase (N/D ratio of 1.05), as did J. Ma. who was undergoing spontaneous diuresis. A.D., who was edema-free, and T.P., who was digitalized, showed reduced excretion of sodium at night.

It is not possible at the present time to establish a cause and effect relation between filtration rate and sodium excretion in either normal subjects or subjects in congestive failure, since other factors affecting tubular reabsorption of sodium remain unanalyzed.

There was no correlation in these patients between the presence of edema and the absolute level of the filtration rate. Four of the 6 patients with edema had filtration rates above the critical level of 70 to 80 cc suggested by Merrill and Cargill(9), while one of the edema-free patients had a filtration rate of only 56.6 cc. The highest filtration rates, 111 and 121 cc, were observed in the 2 patients with edema who were undergoing diuresis.

Brod and Sirota(10) report that the $C'_{CR'}/C_{IN}$ ratio averages close to 1.0 in normal subjects, exceeding 1.0 in patients with renal disease. In all our patients in congestive failure with edema, and in 2 undergoing diuresis, this ratio was significantly below 1.0. Despite this discrepancy between the absolute values of C_{CR} and C_{IN}, the N/D ratios for C_{IN} and C_{CR} were almost identical, indicating that C_{CR}, although not a reliable measure of the absolute value of the filtration rate, is useful for following changes in this function over short periods.

Summary. 1. Diurnal variations of renal

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TABLE I.

Renal Function During Waking and Sleeping Hours in 10 Subjects with Congestive Heart Failure. Clearance Data Are Corrected to Body Surface Area of 1.73 sq. m.

Patient	Age	Diagnosis	Avg urine flow, cc/min.		Avg inulin U/P ratio‖		Average clearance‖ Inulin, cc/min.		'Creatinine', cc/min.		Avg sodium excretion,‖ μEq/min.		N/D ratios Urine flow	Inulin U/P	C_{IN}	C_{CR}	Sodium excretion	C_{CR}/C_{IN}
			Day	Night	Day	Night	Day	Night	Day	Night	Day	Night						
									Persistent edema									
J.Me	49	RHD	.31	.46	260	244	92	107	72	79			1.48	.94	1.16	1.10		.77
A.	39	,,	.40	.38	262	286	100	106	65	69			.95	1.09	1.06	1.06		.65
F.	49	,,	.49	.69	101	81	48	55	37	42	2	4	1.40	.80	1.15	1.13	1.54	.77
E.*	42	,,					94	111	69	79					1.17	1.15		.75
G.	52	CP	.36	.54	82	64	36	48			22	34	1.50	.78	1.35		1.54	
J.	59	ASHD	.67	.63	173	171	92	103	75	83	68	72	.93	.99	1.12	1.12	1.05	.81
									Edema-free or during diuresis									
J.T.†	52	RHD	.48	.50	156	144	74	74	70	71			1.03	.92	1.00	1.01		.95
A.D.†	68	ASHD	.45	.34	145	145	57	52	59	57	33	24	.76	1.00	.91	.97	.72	1.06
T.P.‡	60	,,	2.52	2.36	51	45	111	101	82	83	270	243	.94	.88	.91	1.01	.90	.78
J.Ma§	67	,,	.81	.72	186	196	121	123	98	95	83	102	.89	1.05	1.02	.97	1.24	.79

RHD = Rheumatic heart disease.
CP = Cor pulmonale.
ASHD = Arteriosclerotic heart disease.
* Urine flow and inulin U/P ratios are not recorded in this subject because of the administration of a mercurial diuretic the day before study.
† Free of peripheral edema. Left ventricular heart failure was present, as manifested by dyspnea, orthopnea and basilar pulmonary rales.
‡ Undergoing rapid diuresis following digitalization.
§ This patient was studied during slow spontaneous diuresis.
‖ Values to nearest whole number.

function were studied by means of a constant infusion technic in 10 subjects with congestive heart failure. 2. In contrast to normal subjects, urine flow, sodium excretion and filtration rate increased during sleep in 6 patients with persistent peripheral edema. Four subjects with congestive heart failure who were edema-free or undergoing diuresis showed a diurnal pattern with respect to urine flow and filtration rate similar to that seen in normal subjects. 3. The endogenous 'creatinine' chromogen clearance is substantially lower than the inulin clearance in patients with congestive heart failure and edema.

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Effect of Certain Amines on the Blood Pressure of Normotensive and Hypertensive Rats.* (17979)

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Differences in the usual degree of response of the blood pressure to various pressor substances in experimental hypertension have been described(1,2). Tyramine, epinephrine, pitressin, and renin appear to be more vasoactive in hypertensive dogs and rabbits than in normotensive animals. In the present investigation the pressor responses of isoamylamine, phenethylamine, tyramine, arterenol, and epinephrine have been studied in normal rats and rats with experimental renal hypertension. It was found that the degree of pressor response in the two groups varied with the amine tested. In hypertensive rats isoamylamine showed less, phenethylamine and tyramine the same, and arterenol and epinephrine more effect on blood pressure than in normotensive rats.

Method. Albino rats, of approximately 200 g, were anesthetized by intraperitoneal injection of sodium pentobarbital (4.5 mg per 100 g body weight). By a flank incision the left kidney was isolated and the renal artery freed from the vein. A copper wire, 0.48 mm in diameter, was placed along the artery and a nylon ligature tied tightly around both the artery and the wire. When the wire was removed the artery was left partially constricted. About 70% of the operated animals developed an elevation of systolic blood pressure as measured by a photoelectric plethysmograph, the hind leg being occluded by a pneumatic cuff. After a period of at least 3 weeks to allow for recovery from operative trauma, the hypertensive rats were used for pressor assay. The rat, either hypertensive or normotensive, was again anesthetized and a tracheotomy performed to maintain a patent airway. The right femoral artery was exposed and a saturated solution of novocaine applied to it to prevent constriction. A curved 23 gauge needle connected to a Hamilton optical manometer was inserted into the artery through a small incision. The left femoral vein was isolated and cannulated for the intravenous administration of the substance to be studied. After stabilization, this preparation exhibited levels of blood pressure which were constant within the limits of $\pm$ 5 mm Hg for over 2 hours. The minimal quantity of each amine necessary to produce a pressor response in the normotensive rat was determined.

Results. The following amounts of free base, in gammas per 100 g body weight of rat, produced a "minimal" reproducible pres-

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TABLE I.
Elevation of Blood Pressure of Normotensive and Hypertensive Rats After Graded Doses of Pressor Amines.

Amine	Dose, γ/100 g	Normotensive Rise of B.P.* (mm Hg) No.	High	Avg	Low	Hypertensive Rise of B.P.* (mm Hg) No.	High	Avg	Low
Isoamylamine	80	8	25/25	20/18	15/11	6	10/ 9	6/6	0/0
	160	8	35/33	30/27	18/17	7	14/14	11/ 9	9/8
	320	8	52/51	44/37	40/35	17	35/28	22/16	7/7
Phenethylamine	6.6	7	22/14	17/10	12/ 8	14	36/24	19/14	12/ 4
	13.2	6	23/22	21/15	19/11	9	32/27	26/21	20/15
	26.4	5	43/30	36/27	22/21	11	53/37	34/22	17/10
Tyramine	4.8	9	16/19	14/12	11/ 5	13	23/19	14/11	4/3
	9.6	8	34/20	27/18	19/14	11	39/29	28/22	20/13
	19.2	8	63/37	45/34	29/17	11	52/37	45/36	36/38
DL-arterenol	.06	10	17/12	12/ 9	7/8	12	37/34	35/27	25/24
	.12	12	35/25	26/19	18/11	5	102/57	77/46	47/35
	.24	8	60/46	52/41	44/32	6	121/46	85/47	48/30
L-epinephrine	.06	7	24/14	17/11	9/8	8	42/32	29/23	19/ 9
	.12	8	31/23	23/19	15/13	6	70/49	57/27	36/16
	.24	8	52/35	39/26	21/ 8	6	135/54	75/35	35/17

* "High" indicates the second largest rise; "Avg" the arithmetical mean; "Low" the smallest rise but one. The first figure is the systolic, the second the diastolic rise. All injections are included in these calculations.

sor response: isoamylamine—80.0, phenethylamine—6.6, tyramine—4.8, DL-arterenol—0.06, and L-epinephrine—0.06. A series of responses was obtained for "minimal," twice "minimal" and 4 times "minimal" dose in both the normotensive and hypertensive animals (Table I). The results given are the average values obtained using at least 5 normal and hypertensive rats for each amine. One-half to three-fourths of the "minimal" dose usually elicited inconstant responses.

The minimal reproducible pressor response to each compound studied in the normal rat was approximately the same; the quantity of the base required to produce this response was, however, quite different. With increasing dosage the pressor response seemed to follow a linear progression. When the normotensive series was compared to the hypertensive series a striking difference was noted. In the case of arterenol rats with an elevated blood pressure were more than twice as vasosensitive as normal rats; similar alterations in response to epinephrine were seen principally in systolic pressure. Conversely, hypertensive rats were found to be less than half as sensitive to isoamylamine as the animals with normal blood pressure. However, in the case of phenethylamine and tyramine, the pressor response in the hypertensive group was approximately the same as in the normotensive series.

The duration of the rise of blood pressure was relatively short in normal rats, the effect usually being over in approximately 2 minutes, with certain notable exceptions. Diastolic pressure remained elevated 10 mm Hg or more for 5 minutes after 23% of the injections of phenethylamine, 19% of the injections of tyramine, 12% of the injections of arterenol, 8% of the injections of epinephrine and 7% of the injections of isoamylamine. In hypertensive rats prolongation of the response occurred after approximately the same number of injections of tyramine and epinephrine, while in the case of arterenol and isoamylamine about half as many injections caused prolonged diastolic elevation. Only 8% of injections of phenethylamine produced this effect. None of these substances induced tachyphylaxis.

Discussion. Numerous comprehensive stud-

ies have been made in an attempt to discover a relation between pressor response and chemical structure, carried out on many isolated organ preparations and animals(3). However, no thorough reports contrasting the response of normotensive and hypertensive animals has appeared. Such a correlation would seem to be of value in a search for the possible site of metabolic dysfunction in hypertension. In the present investigation the effect of representative compounds has been utilized: isoamylamine—a branched chain aliphatic amine, phenethylamine—an aromatic amine, tyramine—a phenolic aromatic amine, arterenol—a catechol derivative with an aliphatic hydroxyl group. In the case of isoamylamine, normotensive rats gave the usual pressor response. However in hypertensive rats a notched curve was obtained—an initial increase, a temporary fall, and a secondary increase in blood pressure. This difference has also been noted in comparison of normotensive and hypertensive patients, the latter in some cases showing only a decrease in blood pressure when isoamylamine was injected intravenously. Due to inherent errors in the bioassay method and the progressive nature of hypertension fine differences in structure may not be appreciated but the more important ones can be detected by procedures such as those here reported.

Explanation of these results is difficult in the light of present knowledge. Increased sensitivity of hypertensive animals to pressor agents is explicable upon application of known laws relating peripheral resistance to arteriolar diameter; when arterioles are already constricted, further constriction by a drug causes much greater changes in resistance than when they are dilated. The results found with arterenol are therefore in agreement with this hypothesis. The paradoxical effect of isoamylamine is not. One possible explanation involves the substitution of a substance with slight pressor activity for one with marked activity. If renal hypertension in the rat is accompanied by circulating pressor substances of considerable vasoactivity, "flooding" the circulation with a competitive substance of less activity would give rise to a lessened response. Isoamylamine may act in this manner, although a cardiac effect may be important.

Summary. The pressor response of various amines in anesthetized normotensive and hypertensive rats have been studied. The two groups of animals responded differently to different amines. Whereas phenethylamine and tyramine elicited the same degree of elevation of blood pressure in normotensive and hypertensive rats, arterenol and epinephrine caused greater responses and isoamylamine effected much smaller responses in the hypertensive animals.

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The Use of Radon Seeds to Produce Deep Cerebral Lesions.* (17980)

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Experimental neurophysiology has long made use of the classical method of ablation of various nervous structures and observation of subsequent alteration in function to evaluate the physiological significance of these structures. No problems are encountered when the structures lie on the surface of the brain or spinal cord. When they are deep in the brain substance, however, difficulties are presented in localization and mode of destruction. Although several different technics are available for accomplishing these things in the depths without disturbing superficial structures beyond the small lesion necessary to introduce an instrument, they all have attendant features which are not entirely satisfactory. Among the technics used in the past are injection of chemical substances(1),

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destruction with a knife(2), removal by suction(3), thermocoagulation(4), freezing(5), and electrolytic destruction(6).

In recent years, the method most commonly used has been the electrolytic one, where a stigmatic electrode (anode) is placed deep in the brain and an electric current (DC) passed between it and a large remote electrode (cathode). This method has several disadvantages: *viz.*, there may be vascular spread from a large lesion made at one sitting; the shock of a quickly produced lesion may be fatal to the animal; the size of the lesion produced is difficult to control and because of the danger of vascular spread can never be large. Therefore, we have adapted a method which produces a lesion more gradually, that is with less shock, and which permits the production of a large lesion with a single puncture, obviating the necessity of inserting multiple electrodes into the brain. It is the method first used by Edwards and Bogg(7), who worked on dogs and made their lesions in the corpus striatum. It seemed worth while to us to modify the method and explore its suitability for producing stereotaxically controlled lesions.

Our experiments were done on monkeys (*Macaca mulatta*) under nembutal anesthesia. The animal's head was fixed in a stereotaxic instrument† and draped for aseptic surgery. The needle-holder of the instrument, set to hit the desired structure, introduced a hollow needle into the brain. A gold radon seed was carried just inside the tip of the needle and a stylet was used to deliver the seed into the brain. The surgical procedure consisted of incising the skin, trephining a hole in the skull approximately 6 mm in diameter, and cutting a piece of the dura no more than 1.5 x 1.5 mm. Following the operation, the monkeys were observed for changes in neurological status. All the animals in this study had needles directed into the midbrain 30 degrees anterior to the traditional Horsley-Clarke plane, which placed the needles roughly in the long axis of the brain stem. We will cite the protocol of one of our monkeys to illustrate the findings in our series: A female weighing 2.9 kg was observed following the placement of seed 2 mm in length containing 2.82 mc of radon.

On the day following operation, her left pupil was found to be fully dilated and her head tilted so that it rested on her right shoulder. A postural tremor was observed at the elbow and shoulder of the right upper extremity. This tremor was not continuous but occurred in bouts. On the second post-operative day, both pupils were dilated and both upper lids were ptosed. Her head remained tilted and there was deviation of the upper spine to the right. She moved with a squatting posture and kept circling to the right. Pain sensation was absent in the right leg and diminished in the right arm. An occasional bout of postural tremor was observed in the left hip and knee. There was weakness in the right arm and right leg. On the third post-operative day all tremor disappeared. On the fourth post-operative day she was observed to sit with her head hanging. The weakness of the right arm and leg had progressed, but motor function in the left extremities remained good. Both knee jerks were a little brisk, but there was no clearly defined spasticity. The left extremities, which she used to defend herself, did not appear to have any dyssynergia. No pain sensation was appreciated in the right arm and leg. Because of these definite neurological changes,

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† Lab-Tronics, Inc., Model No. G-101.

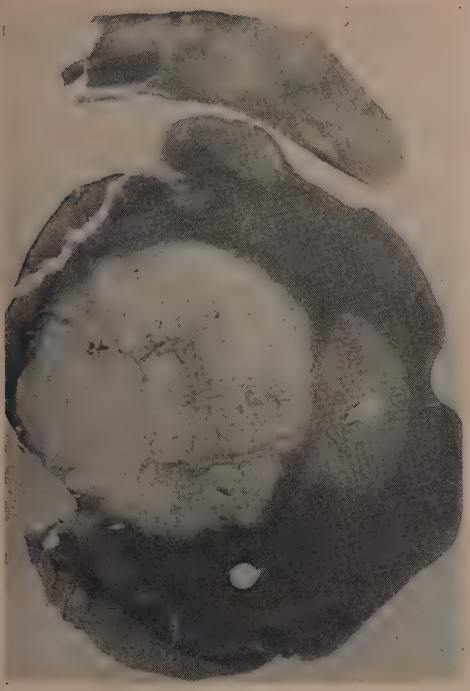

FIG. 1.

the monkey was sacrificed on the fourth postoperative day, and the brain perfused with saline followed by 10% formalin. X-ray pictures were made of the skull to show the position of the radon seed, and then the brain was removed for study.

Histological examination of this brain showed a spherical lesion (Fig. 1) which measured 1 cm in diameter, having its center in the midbrain at the level of emergence of the third nerves. The lesion destroyed all of the left and most of the right oculomotor nerve, all of the red nucleus and decussation of the superior brachium and the medial half of the left substantia nigra. The medial tegmentum adjacent to the substantia nigra and a large part of the lateral mesencephalic tegmentum on the left side, and the ventral portion of the central grey were also destroyed. The lesion extended cephalad on the left, destroying structures lying dorsal to the mammillary bodies and medial to the subthalamic nucleus, including the fasciculus thalamicus and median center. Caudally, destruction extended into the tegmentum of the pons at the level of the inferior colliculi. The lesion appeared to be an acute necrosis without vascular spread, and the neurones which surrounded the lesion appeared normal. The zone of cellular reaction in the surrounding brain was very small. The destroyed area thus had a "punched-out" appearance because the necrosis within the lesion was so complete. Except for the persistence of short stubby remnants of some of the larger blood vessels, no structure within the lesions could be identified.

Thus the following features are salient in this method of making a lesion. First, a large lesion can be produced with a single small wound of entry. Second, the lesion is produced so slowly that nervous tissue is not subjected to abrupt change, with resultant "shock." Hence, a larger lesion is possible than can be produced in one sitting by electrolytic means. Third, the lesion can be located easily during life by X-ray (the radon seed is radio-opaque). And fourth, the size of the lesion produced can easily be controlled by choosing the dose of radon.

For these reasons, the method outlined above seems to offer definite usefulness in the production of animals with deep chronic lesions. We would also like to suggest this method to the neurosurgeon, especially since there has recently appeared on the market an adequately designed stereotaxic instrument for man.‡ We see no reason why the stereotaxic technic cannot be used in the attack on human cerebral dysfunction where destruction of certain tracts or nuclear structures deep in the brain are considered to be of value. In fact, stereotaxically placed radon seeds might offer an approach to the treatment of deep neoplasms of the thalamus or basal ganglia.

Summary and conclusions. 1. Stereotaxically placed radon seeds in the brains of monkeys produced well controlled lesions suitable for studying certain aspects of cerebral physiology. 2. The technic described, which requires only a minor surgical procedure, should be considered for producing deep lesions and for attacking deep tumors in the human brain.

‡ Lab-Tronics, Inc., Model No. G-111.

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Effects of Prolonged Exposure to Cold on the Vitamin A Requirement of the Rat.* (17981)

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Available data indicate that requirements for a number of nutrients are markedly increased under conditions of low environmental temperature. This is particularly true for some of the water-soluble vitamins. An increased requirement for thiamine(1,2), pyridoxine(3) and ascorbic acid(4) has been demonstrated following prolonged exposure to low environmental temperatures. In the present communication data are presented on the effects of low environmental temperature on the vitamin A requirement of the rat. Animals were fed purified rations deficient in vit. A and their rate of depletion and length of survival determined under cold room and room temperature conditions.

Procedure and results. The vit. A-free ration employed in the present experiment consisted of sucrose, 60%; casein,† 25%; salt mixture,‡ 5%; and cottonseed oil (Wesson), 10%. To each kg of the above were added the following synthetic vitamins: thiamine hydrochloride, 40 mg; riboflavin, 40 mg; pyridoxine hydrochloride, 40 mg; calcium pantothenate, 80 mg; nicotinic acid, 60 mg; ascorbic acid, 100 mg; biotin, 5 mg; folic acid, 10 mg; p-aminobenzoic acid, 400 mg; inositol, 800 mg; vit. B_{12},§ 60 γ; 2-methylnaphthoquinone, 10 mg; vit. D,‖ 400 U.S.P. units; and choline chloride, 2 g. Each rat also received once weekly 4.5 mg of alpha-tocopherol acetate. The animals employed in the present experiment were male rats of the University of Southern California strain in which the dietary regime of the parents was such as to make the young rats satisfactory for bioassay studies for vit. A. Parents were maintained for at least 2 months previous to breeding and for 14 days after the birth of the litter on Sherman diet B(5) without addition of supplementary lettuce or meat. Litters were cut to 7 at 3 days of age, and on the 14th day mothers and litters were placed on a vit. A-low diet.¶ At 26 days of age the young rats were placed either on the vit. A-free diet indicated above or a similar ration supplemented with 6000 U.S.P. units of vit. A per kg of diet.** Tests were conducted (1) with animals kept continuously in a large walk-in refrigerator at a temperature of 2 ± 1.5°C and (2) under standard laboratory conditions at an average temperature of approximately 23 ± 2°C. The dietary groups consisted of 12 rats each in the room temperature series and 16 animals per group in the cold room series. Rats were kept in individual metal cages with raised screen bottoms to prevent access to feces and were fed *ad lib* the diets

* This paper reports research undertaken in cooperation with the Quartermaster Food and Container Institute for the Armed Forces, and has been assigned number 312 in the series of papers approved for publication. The views or conclusions contained in this report are those of the author. They are not to be construed as necessarily reflecting the views or indorsement of the Department of the Army.

Communication No. 265 from the Department of Biochemistry and Nutrition, University of Southern California.

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† Vitamin Test Casein, General Biochemicals, Inc., Chagrin Falls, Ohio.

‡ Hubbel, Mendel and Wakeman Salt Mixture, General Biochemicals, Inc., Chagrin Falls, Ohio.

§ Vitamin B_{12} Oral Grade Solids, Chas. Pfizer & Co., Brooklyn, N. Y.

‖ HY-DEE Powder, Standard Brands, Inc., New York City.

5. Sherman, H. C., and Campbell, H. L., *J. Biol. Chem.,* 1924, v60, 5.

¶ Similar to U.S.P. XII depletion diet except that commercial casein rather than extracted (vitamin A-free) casein was employed.

** MYVA-DRY Powder, Distillation Products, Inc., Rochester, N. Y.

TABLE I.
Effects of Low Environmental Temperature on the Rate of Depletion and Length of Survival of Immature Rats Fed a Vitamin A-free Diet (10 animals per group).

Initial body wt, g	Values end of depletion period		Avg survival after depletion*		Body wt after 50 days of feeding, g
	Avg age, days	Avg wt, g	Days	Range	
		Cold room series			
55.2	40.4	73.4	4.0 ± 0.5	(2–6)	—
54.6	—	—	—		136.4
		Room temperature series			
53.1	44.3	107.3	20.4 ± 1.4	(12–27)	—
52.8	—	—	—		233.1

* Including standard error of the mean calculated as follows: $\sqrt{\frac{\Sigma d^2}{n}} / \sqrt{n}$ where "d" is the deviation from the mean and "n" is the number of observations.

listed above. Feeding was continued for 50 days or until death, whichever occurred sooner.

Results are summarized in Table I. Data were computed on the basis of the top 10 animals in each group in order to minimize variations in averages due to early deaths, infection and atypical responses on the part of individual rats. The end of the depletion period was arbitrarily set as the 5th day on which animals on a vit. A-free diet showed stationary or decreasing body weight. Findings indicate that rats fed a vit. A-free diet under cold room conditions differed from those fed a similar ration at room temperature in the following respects: (1) rats depleted more rapidly under cold room conditions, (2) body weight at time of depletion was less in the cold room than in room temperature series and (3) length of survival after depletion was significantly decreased in the cold room series. Gain in body weight was also reduced in animals fed the A+ diet under cold room conditions. These rats, however, did not develop xerophthalmia or other manifestations of vit. A deficiency as did animals on the vit. A-free diet. In accordance with previous findings(6) gangrene of the tail tip and edges of the ear was common in virtually all rats in the cold room series with amputations of the entire tail occurring in approximately 20% of the series. In general, however, these effects occurred with equal frequency on both diets employed.

Present findings indicate that prolonged exposure to cold increases the vit. A requirement of the rat. This is indicated by the fact that animals maintained on a vit. A-free diet depleted more rapidly under cold room than room temperature conditions and also by the shorter survival time after depletion of vit. A-deficient rats under conditions of low environmental temperature. A similar decrease in post-depletion survival time has been observed in vit. A-deficient rats following growth hormone administration(7). It is possible, however, that the decreased length of survival after depletion may reflect not a more rapid exhaustion of body stores of vit. A (as was suggested in the case of growth hormone) but rather failure of some physiological mechanism in the vit. A-depleted rat which is essential for resistance to cold.

Summary. Immature rats were fed purified rations deficient in vit. A under cold room (2°C) and room temperature (23°C) conditions. The following results were obtained: (1) rats depleted more rapidly under cold room conditions, (2) body weight at time of depletion was less in the cold room than room temperature series, and (3) length of survival after depletion was significantly decreased in the cold room series.

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Oxybiotin in the Bacterial Deamination of Aspartic Acid. (17982)

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Oxybiotin has been shown to have biotin-like activity for a variety of microbial and animal species(1-5). It is now accepted that, in the systems that have been studied, oxybiotin functions as such without conversion to biotin. The statement has been made that, "Since several widely different species can utilize oxybiotin as such, it seems reasonable to predict that oxybiotin can replace biotin in all biological forms"(6).

Among the functions in which biotin more recently has been implicated is that of stimulating the aspartic acid deaminase activity of bacterial cells that have been exposed to M phosphate buffer of pH 4(7-12). The effect of oxybiotin under these conditions has not been reported previously and this paper describes the ability of oxybiotin to replace biotin in the system.

Experimental. Procedures that have been employed in the aspartic acid deaminase experiments have been described in detail(12). Briefly an 18-hour culture of the organism in a yeast extract-tryptone-formate-phosphate medium was centrifuged, washed in water, recentrifuged, suspended in M phosphate buffer of pH 4 at 37°C for inactivation of the aspartic acid system, and neutralized. The effect of various adjuvants on the ability of the organism to deaminate added aspartic

TABLE I.
Protocol for Aspartic Acid Deamination.

	No. 1, ml	No. 2, ml	No. 3, ml	No. 4, ml
Bacterial suspension in pH 4 M phosphate buffer	.5	.5	.5	.5
Inactivated at 37°C for 30 min.				
Na_3PO_4 (saturated)*	.5	—	—	—
Aspartic acid† in Na_3PO_4 (saturated)*	—	.5	.5	.5
H_2O	1.0	1.0	—	—
Biotin‡	—	—	1.0	—
Oxybiotin§ or desthiobiotin‖	—	—	—	1.0
Incubated at 37°C for 30 min.				
Trichloroacetic acid	.5	.5	.5	.5

Centrifuged and ammonia nitrogen determined by Nesslerization on an aliquot of each centrifugate.

* This addition brings the pH to 7.0.

† 266 mg DL-aspartic acid dissolved in 100 ml saturated Na_3PO_4. Calculated to give 0.005 M DL-aspartic acid in the final reaction mixture.

‡ 0.001 γ D-biotin/ml.

§ 0.001 γ or 1.0 γ D-oxybiotin/ml.

‖ 0.001 γ D-desthiobiotin/ml.

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11. Lichstein, H. C., and Christman, J. F., *J. Bact.,* 1949, v58, 565.

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TABLE II.
Summary of Aspartic Acid Deaminase Experiments.

Exp. No.	Ammonia nitrogen formed			
	No supplement, γ	Biotin, γ	Oxybiotin, γ	Desthiobiotin, γ
1	11.8	18.0 (.001 γ)	17.3 (.001 γ)	—
2	11.5	58.5 (.001 γ)	25.0 (.001 γ)	—
3	10.0	51.5 (.001 γ)	16.0 (.001 γ)	—
4	3.5	30.0 (.001 γ)	41.5 (1.0 γ)	—
5	13.0	57.5 (.001 γ)	—	15.0 (.001 γ)

Figures in parentheses indicate the quantity of supplement tested per 2 ml reaction mixture.

acid at pH 7 then was determined according to the protocol given in Table I. A strain of *Proteus vulgaris* was the organism employed. The oxybiotin* (O-heterobiotin)* and the desthiobiotin used were racemic mixtures. The quantities used in this paper are in terms of active D– component employed.

Results and discussion. The results that have been obtained are summarized in Table II. It is apparent from experiments 1-3, where biotin and oxybiotin were tested at equivalent levels, that oxybiotin is active in stimulating the aspartic acid deaminase system, although it is somewhat less active than is biotin. As demonstrated by experiment 4, when tested at a high level (1.0 γ/tube) oxybiotin can replace completely the functions of biotin in the bacterial deamination of aspartic acid. Desthiobiotin, as the results of experiment 5 indicate, is essentially inactive. Thus it appears that ring closure is essential for activity in the present system as it is in other systems involving biotin that have been studied previously.

Summary. Oxybiotin is less active than biotin in stimulating the aspartic acid deaminase system of bacterial cells that have been inactivated by exposure to M phosphate buffer of pH 4. In sufficient amount, however, oxybiotin duplicates completely the stimulation produced with biotin.

* Supplied through the courtesy of the Hoffmann-La Roche Laboratories.

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Effect of Hepatectomy upon the Analgetic Action of 1 Methadone.* (17983)

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While it may be considered that the liver is probably the chief site of detoxification of 1 Methadone, it was of interest to test this hypothesis. It was proposed to determine this by examining the effect of partial hepatectomy upon the duration of analgesia following a standard dose of the drug administered to rats.

* Assisted in part by a grant from Hoffmann-La Roche, Inc., Nutley, New Jersey.

Methods. Male albino rats (Red Bank) weighing 250 to 350 g were used. Analgesia was measured using a modification of the D'Amour Smith method(1,2) in which radiant heat is applied to the terminal portion of the rat's tail. One of the chief features

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2. Bonnycastle, D. D., and Molland, J., *Fed. Proc.*, 1948.

of this modification is that the rats determine by their original reaction times, the maximal allowable stimulation at any one time. This restriction permits the repeated application of the stimulus without any great hazard of producing tissue injury.

After preliminary experiments, it was decided that a standard intraperitoneal dose of 1.5 mg/kg 1 Methadone was suitable, because this dose was sufficient to abolish a reaction to the stimulus within the period of stimulation in all cases, and yet the animals had returned to normal within two hours. The experimental group of animals, 18 in number, was treated with this standard dose of 1.5 mg/kg of 1 Methadone, and the effect of the drug observed for 3 hours.

The next day, partial hepatectomy was carried out upon 9 of these rats, following the method of Higgins and Anderson(3). It was calculated that for this group of animals, 72 to 81% of the total liver had been removed. A mock operation, consisting of laparotomy alone, was performed on the remaining 9 rats of the group. Five days postoperatively the animals were tested with 1.5 mg/kg intraperitoneally of 1 Methadone and subsequently on the 15th postoperative day. The differences in duration of analgesia were compared statistically by standard methods.

Results. In the control series the animals all exhibited a failure to react to the stimulus following the administration of the 1 Methadone but all had returned to their previous levels of reaction within 2 hours.

It is seen in Fig. 1 that in response to the standard dose there is a difference in the incidence and duration of analgesia between the unoperated normal control values and the post-laparotomy values. This difference in the duration is significant. The effect of the standard 1.5 mg/kg dose of 1 Methadone upon the partial hepatectomized group and the control laparotomy group is shown in Fig. 2. Here the difference in duration of analgesia is highly significant, as also is the difference between the normal control and partially hepatectomized group.

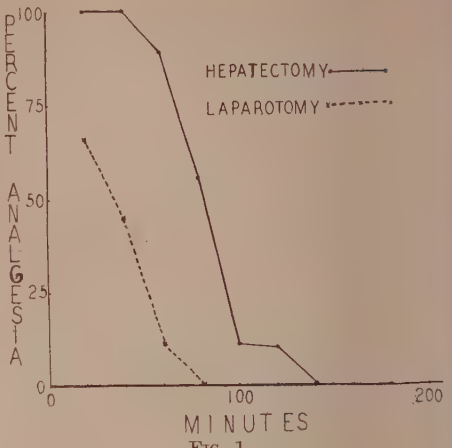

FIG. 1.

Fig. 3 presents the values obtained 15 days postoperatively, and shows that the two groups, *i.e.*, the laparotomy and partially hepatectomized groups, react to the drug in essentially the same manner as before the operations.

Discussion. The effect of partial hepatectomy in increasing the duration of the action of 1 Methadone as tested in this manner is of interest. It does suggest that the liver is of great importance in the metabolism of the drug. At the time these experiments were completed, a report by Chen-Yü Sung *et al.*(4) appeared in which essentially the same findings were obtained for d,l Methadone. These investigators also stated that *in vitro* studies led to the conclusion that the liver was the chief site of metabolism of the drug.

It is of interest that there was a significant degree of difference between the effect of the drug in the laparotomy-control group and the effect on the same animals before operation. This again emphasizes the fact that in such experiments control animals should be used upon which sham operations have been carried out.

While at 15 days postoperatively the livers of the partially hepatectomized group had apparently regenerated sufficiently so that the response was similar to that obtained in

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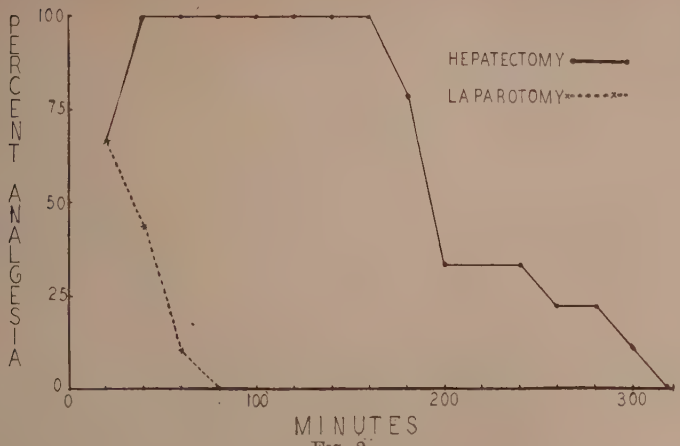

FIG. 2.

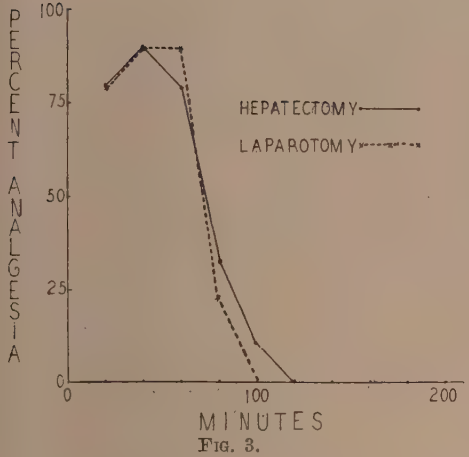

FIG. 3.

the control period, it cannot be taken to indicate that complete regeneration has taken place. In fact it has been stated that this takes 28 to 30 days(3) or possibly longer.

Summary and conclusions. A comparison of the analgetic response of 1.5 mg/kg intraperitoneally of 1 Methadone in normal, sham-operated and partially hepatectomized rats was carried out 5 days postoperatively, and 15 days postoperatively.

These experiments suggest that the liver is the chief site for the destruction of 1 Methadone.

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Persistence of Brunhilde Poliomyelitis Virus in Rodent Brain without Evidence of Adaptation.* (17984)

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Although all evidence indicated that non-Lansing types of human poliomyelitis virus would be difficult to adapt to nonprimate hosts, it seemed worthwhile to reinvestigate the problem using a virus of high titer and a reasonably large number of animals. The method of alternating passage between rhesus monkeys and other animals was employed in addition to passage from rodent to rodent.

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The former method of alternation of the passage of a virus between hosts of known susceptibility and hosts which were less susceptible has apparently achieved success in the adaptation of certain viruses to less susceptible hosts(1-3).

Material and methods. Brunhilde virus pool II(4) with an intracerebral titer of 0.8 ml x $10^{-5.9}$ was used in all experiments. This virus is antigenically distinct from viruses of the Lansing type. Rhesus monkeys of approximately 5-6 lb in weight were inoculated intrathalamically with 0.8-1.0 ml of 20% brain emulsions. The inoculum was divided equally between left and right thalami. Monkeys were observed daily and were sacrificed at the onset of symptoms or after 30 days when no symptoms were observed. Histological confirmation of the diagnosis was made of all paralytic and non-paralytic monkeys. The cotton rats *(Sigmodon hispidus hispidus)* (Say and Ord), golden hamsters *(Cricetus auratus)*, meadow voles *(Microtus pennsylvanicus pennsylvanicus)* (Ord), and deer mice *(Peromyscus leucopus noveboracensis)* (Fischer) were of the stock of Tumblebrook Farm, Brant Lake, New York. Swiss albino mice were obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine. The cotton rats were approximately 5 weeks old, and the other rodents about 3 weeks old at the time of inoculation. Routinely all rodents were inoculated intracerebrally; the quantity of the inoculum employed for cotton rats and hamsters was 0.05 ml and for voles and deer mice, 0.03 ml. Certain samples of central nervous system tissue when inoculated as a 20% suspension caused the immediate death of up to 30% of rats. This non-specific death rate was sometimes reduced practically to zero by dividing the inoculum between both sides of the brain.

In all experiments a sufficient number of groups of rats and hamsters was inoculated so that the effects of each suspension could be observed in 25-30 animals for a period of at least 6 weeks. Excluding animals dying immediately or on the first day after inoculation, a total of 732 cotton rats and 97 hamsters was kept under observation. Voles were inoculated in groups of 12 to 18, and deer mice in groups of 18 to 24. A total of 256 of the former, 52 of the latter were inoculated.

Experimental. (a) *Alternation of passages*: The alternation of passages between cotton rats and rhesus monkeys was initiated by the intracerebral inoculation of cotton rats with 20% Brunhilde virus. On the 1st, 3rd, and 5th days after inoculation, 6 rats were sacrificed, and their brains pooled and tested for virus by the inoculation of 2 rhesus monkeys with each pool. Virus was found to be present in each emulsion of rat brains as indicated by the onset of paralysis and subsequent demonstration of typical histological lesions in the inoculated monkeys.

A 20% emulsion was made of the pooled lumbar and cervical enlargements of the monkeys inoculated with the pool of rat brains which had been harvested on the 5th day. This monkey cord emulsion was inoculated into other groups of rats. Six rats of the latter groups were killed and their brains harvested and pooled on the 5th and 10th days after inoculation. Virus was demonstrated to be present in both pools by the inoculation of rhesus monkeys. The remaining rats in this group were kept under observation for 6 weeks. This alternation of passage of virus from the brains of rats sacrificed on the 5th and 10th day back to monkeys and into rats again was continued for 5 cycles. It was clear (Fig. 1) that virus could be recovered from the brains of rats up to the 10th day after inoculation. The rats from which virus was recovered were, however, symptomless. Brains of 9 rats were examined histologically 10 days after inoculation with 20% Brunhilde virus. No lesions, other than could be accounted for by the inoculations, were found. During the 6 week observation period, any rat show-

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FIGURE SHOWING PASSAGES OF BRUNHILDE VIRUS IN RHESUS MONKEYS AND COTTON RATS

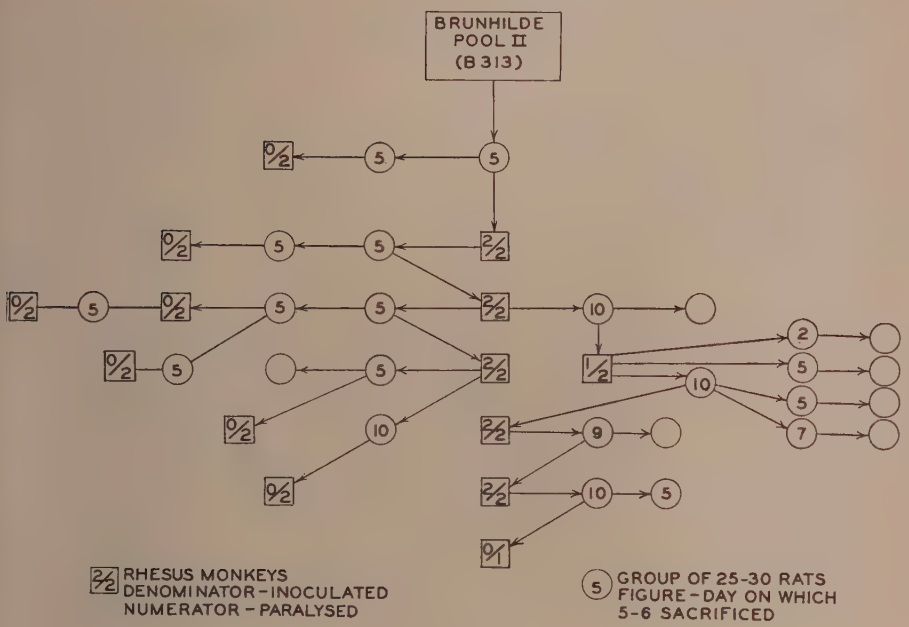

FIG. 1.

ing the least abnormality was sacrificed. In 18 of these sacrificed rats, the brain was passed to other rats, and the cord from 5 of them was examined histologically. In 4 other sacrificed rats, histological examination of the cord was made. In none of these animals was there any visible evidence of virus proliferation.

An experiment similar to the above was set up in which alternation of passage of virus between rhesus monkeys and hamsters was carried out. Virus was recovered in monkeys from the brains of hamsters up to the 5th day after the inoculation of virus. No later tests were made. This experiment was abandoned in the 2nd cycle because of difficulties in obtaining sterile brain emulsions from hamsters. No evidence of virus activity was observed in any of the inoculated hamsters.

(b) *Rodent to rodent passages.* After it had been established that Brunhilde virus was present in cotton rat brains up to the 10th day after inoculation, 20% emulsions of brains of rats killed on the 5th, 9th, or 10th days were inoculated into other rats as shown in Fig. 1. Virus was not recovered in rhesus monkeys after more than one cotton rat passage, nor was there any evidence of disease in the rats themselves.

In addition to these direct passages of 20% brain suspensions, the following passages were made. A pool was made of the cervical and lumbar enlargements from monkeys which had become paralysed after inoculation with cotton rat brains containing virus. The supernate of this pool was concentrated ten times by ultracentrifugation,‡ and 12 rats were inoculated with the concentrate. Five groups, each of 6 rats, were given 3 to 5 inoculations intracerebrally and intraperitoneally daily or on alternate days with suspensions

‡ This preparation was made by Dr. C. E. Schwerdt.

of monkey cord or rat brain containing Brunhilde virus. Brunhilde virus was diluted in 10% normal rat brain supernate in another experiment, and inoculated intracerebrally into 24 rats. By none of these methods was any adaptation accomplished.

Brains of hamsters sacrificed on the 5th day after inoculation with Brunhilde virus were passed to other hamsters. Although Brunhilde virus was recovered in rhesus monkeys after a 5 days sojourn in the brains of symptomless hamsters, there was no evidence of transmission of the virus to other groups of hamsters.

Voles were inoculated with 20% Brunhilde virus. Unfortunately, voles appear to have a high non-specific death rate under laboratory conditions. The medulla and cord from 19 voles which were sick or died after inoculation of Brunhilde virus were examined histologically. In none of these animals was there any evidence of virus proliferation. Passages were made to groups of 10-20 voles with the brains of 5 of the dead or sick voles, with evidence of bacterial infection in 2 cases.

The susceptibility of the vole to Lansing virus does not appear to exceed that of the cotton rat. In 2 titrations of Lansing virus derived from monkey cord and employing 10-fold dilutions with 5-7 voles per dilution, there were no paralyses above 10^{-3}. This material (Pools IVa and IVa-1) had a median titer of $10^{-3.5}$(5), in cotton rats. A Lansing pool of mouse origin, J. Bell(5) with a mean titer of $10^{-4.6}$ in cotton rats was 3.7 in a single titration in voles using 12 animals to a dilution. The titration, however, was very irregular since even at the lower dilutions, 100% paralysis was not obtained. The test was further complicated by a number of deaths not preceded by observable paralysis. In view of the high non-specific death rate among voles, these deaths were excluded from the titration which ran as follows: $10^{-1.5}$ 8/10; $10^{-2.5}$ 4/7; $10^{-3.5}$ 6/10; $10^{-4.5}$ 5/8. It seems likely, however, that although the end point was not definitely reached, it probably would not have exceeded that in cotton rats.

A similar experiment to the above was made with deer mice. There was no evidence of virus adaptation to these animals.

In view of the persistence of virus in the brains of hamsters and cotton rats up to the 5th and 10th day respectively after inoculation, an experiment was done to see if the virus could be maintained in mouse brains. Thirty-two Swiss albino mice were inoculated intracerebrally with 0.02 ml of 20% Brunhilde virus. This quantity of inoculum was chosen so that the dilution factor in the mouse brain would be of the same order as that of virus inoculated into cotton rat brains. Two groups of symptomless mice were sacrificed on the 5th and 10th days after inoculation. The brains from each group were pooled separately and each of the four were inoculated into 2 rhesus monkeys. All the inoculated rhesus monkeys except one developed symptoms of poliomyelitis indicating that Brunhilde virus had remained present in mouse brains up to 10 days after inoculation.

Summary and conclusions. Adaptation of Brunhilde virus to cotton rats or hamsters was not achieved by the method of alternation of passage between these animals and rhesus monkeys.

Brunhilde virus was found present in the brains of symptomless hamsters up to 5 days after intracerebral inoculation and up to 10 days after intracerebral inoculation of cotton rats and Swiss albino mice (no tests at longer intervals were made). There was no evidence of the virus persisting after more than one cotton rat or hamster passage, and there was no histological evidence of virus proliferation. This is highly suggestive that no multiplication had occurred in the brains of these animals. This persistence of virus in symptomless animals is similar to that observed by Smith(6) in attempts to establish St. Louis encephalitis in rats and guinea pigs.

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p-Anisaldehyde-Thiosemicarbazone in Treatment of Experimental Murine Tuberculosis.* (17985)

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Since Domagk and his coworkers(1) described the antimycobacterial properties of various thiosemicarbazones, these compounds have undergone laboratory and clinical investigation(2-9). This is a preliminary study of the antimycobacterial activity of one of the more promising thiosemicarbazones described by Domagk(1).

Materials and method. In vitro testing. p-anisaldehyde-thiosemicarbazone was prepared for *in vitro* testing as follows: A 0.50% solution of the compound was made up in hot 50% alcohol. This was diluted in Dubos' medium(10) to suitable concentrations, inoculated with *Mycobacterium tuberculosis* $H_{37}RV$ and with *Mycobacterium tuberculosis* B1 and incubated for 14 days. The results indicated that the compound had far greater *in vitro* activity than streptomycin or para-aminosalicylic acid(11). It prevented the growth of both strains of the tubercle bacillus at a concentration of 1.0 μg%.

In vivo testing. Fifty dba mice, weighing approximately 25 g, were infected intravenously with 0.5 cc of a 7-day culture of $H_{37}RV$ grown at 37°C in Dubos' Tween-albumin medium. Twenty-four hours after the infection the mice were divided into 2 groups of 25 each. The first control group was untreated. The second group received the thiosemicarbazone mixed in ground mouse food in 1% concentration *ad libitum.*

Thirty days after the infection all the untreated controls were dead. At autopsy these animals all showed extensive ++++ gross pulmonary tuberculosis. No other gross lesions were observed. Two of the treated animals died within 4 days of infection from causes other than tuberculosis. Fourteen of the remaining treated animals were sacrificed at the end of 30 days. The autopsies revealed no gross pulmonary tuberculosis nor other lesions, except for the spleens, which were markedly enlarged. The remaining 9 treated mice, which appeared to be in good health, were sacrificed 75 days after infection. The autopsies revealed an average of only one plus or very slight pulmonary tuberculosis. The liver and kidneys appeared normal. The spleens, again, were enlarged, as in the treated animals autopsied after 30 days.

Summary. 1. One mg % of p-anisaldehyde-thiosemicarbazone inhibits the growth of the $H_{37}RV$ and Bl strains of *Mycobacterium tuberculosis* in Dubos' medium.

2. Mice infected with the $H_{37}RV$ strain and fed 1% p-anisaldehyde-thiosemicarbazone in their diet had no gross tuberculosis when sacrificed at the end of 30 days, when all controls were dead of the disease. Infected

* Aided by a grant from the Nepera Chemical Co,. Inc.

† Deceased.

We extend our appreciation to the Nepera Chemical Co., Inc. for supplying the drug, and to Dr. Charles J. Duca for conducting the *in vitro* work.

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8. Spinks, A., *Brit. J. Pharmacol. and Chemo.,* 1949, v4, 254.

9. Hoggarth, E., Martin, A. R., Story, N. E. and Young, E. H. P., *Brit. J. Pharmacol. and Chemo.,* 1949, v4, 254.

10. Dubos, R. J., *Amer. Rev. Tuberc.,* 1947, v56, 334.

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mice, sacrificed after 75 days of treatment with p-anisaldehyde-thiosemicarbazone, showed only one plus gross pulmonary tuberculosis.

3. It may be concluded that p-anisaldehyde-thiosemicarbazone is an effective antimycobacterial agent in the mouse as well as *in vitro*.

We extend our appreciation to the Nepera Chemical Co., Inc., for supplying the drug, and to Dr. Charles J. Duca for conducting the *in vitro* work.

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A Comparative Study of Susceptibility of Tubercle Bacillus ($H_{37}RV$) to Aureomycin, Streptomycin, and Para-aminosalicylic Acid.* (17986)

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Aureomycin, an antibiotic derived from *Streptomyces aureofaciens,* has been effective in the treatment of a wide variety of bacterial, rickettsial and virus diseases(1). For the purpose of exploring its possible usefulness as an antibiotic agent in tuberculosis, experiments with this agent were carried out to compare it with streptomycin and para-aminosalicylic acid. The latter 2 antibiotics are known to be active against the tubercle bacillus(2,3), while only brief mention of the activity of aureomycin against this organism has been made(4,5,6).

In vitro experiment. Sterile solutions of streptomycin and aureomycin were prepared in Dubos' Tween-albumin medium. The para-aminosalicylic acid was dissolved in a saturated solution of $NaHCO_3$ and then brought to pH 7.0 with M/15 phosphate buffer. The solution was Seitz-filtered and then added to Dubos' medium. The concentrations of streptomycin, aureomycin, and para-aminosalicylic acid tested, ranged from 0.1 to 30.0 μg/cc of medium. The tubes containing 5 cc of each dilution were inoculated with 0.1 cc of a 10-day $H_{37}RV$ culture from Dubos' medium. The concentrations of aureomycin were maintained by adding suitable amounts of aureomycin every 24 hours for the duration of the experiment, since its rate of deterioration is fairly constant(1). The amount of growth was graded from ++++, representing a milky growth, to 0, representing no growth. Whenever present, growth was checked by stained smears. Aureomycin compares favorably with streptomycin and para-aminosalicylic acid in inhibiting the growth of the tubercle bacillus *in vitro* (Table I).

In vivo experiments. Seventy-five dba mice, averaging 25 g in weight, were infected intravenously with 0.1 mg of $H_{37}RV$ suspended in 0.2 cc of saline. The mice were then divided into 5 groups of 15 each. Group I was untreated, Group II received 1000 μg/cc of streptomycin, injected subcutaneously, in 0.25 cc of saline every 12 hours. Group III received 1.5% para-aminosalicylic acid incorporated in the diet. Group IV and Group V received 1000 and 5000 μg/cc, respectively, of aureomycin, injected subcutaneously in

* Aided by a grant from the Nepera Chemical Co., Inc., Yonkers, N. Y.

The aureomycin was supplied by the Lederle Laboratories Division of the American Cyanamid Corp.

† Deceased.

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TABLE I.
Inhibition of the Growth of $H_{37}RV$ in Dubos' Tween-Albumin Medium.

Antibiotic	Concentration in μg/cc											
	0.1		0.2		0.5		1.0		2.0		4.0	
	6 days*	15 days	6 days	15 days	6 days	15 days	6 days	15 days	6 days	15 days	6 days	15 days
Aureomycin	++	+++	+++	+++	++	+++	0	0	0	0	0	0
Streptomycin	++	++++	++	++++	+	+++	0	0	0	0	0	0
Para-aminosalicylic acid	+++	+++	+++	+++	++	+++	+	+++	0	++	0	0

* Controls with no antibiotic were fully grown at the end of this period of time.

TABLE II.
Average Gross Tuberculosis in Lungs of dba Mice Treated with Aureomycin, Streptomycin, and Para-aminosalicylic Acid.

Group	Treatment	Avg degree of lung lesions
I	Control—none	3.5+
II	Streptomycin, 2,000 μg/day	0.8+
III	Para-aminosalicylic acid 1.5% in food	2.0+
IV	Aureomycin, 2,000 μg/day	3.4+
V	" 10,000 "	3.9+

0.25 cc of saline every 12 hours. Treatment for all groups was begun 24 hours after infection. Sixty days after infection all surviving mice were sacrificed. Treatment was continued to within 2 days of sacrifice.

At autopsy the lungs, liver, kidney, and spleen were examined grossly and were fixed in Zenker's solution for further histological study. No gross tuberculosis was observed except in the lungs. The appearance of the lesions in the lungs was graded from 0 to ++++(7). It may be seen from Table II that the degree of gross tuberculosis of the lungs in the aureomycin-treated animals was equal to that in the controls.

Microscopically, in the lungs, the lesions appeared as tuberculous broncho-pneumonia. The alveoli were filled with mononuclear cells and the walls were thickened and infiltrated. Only in the streptomycin-treated groups did the pulmonary lesions, when present, contain significant numbers of lymphocytes. In an occasional streptomycin-treated mouse fibrosis was found. Necrosis and caseation were rare and were equally distributed among Groups I, III, IV, and V.

The livers contained perivascular tubercles composed of mononuclear cells, lymphocytes, and a few epithelioid cells. In the spleen the lesions were composed of epithelioid cells and mononuclears, usually surrounded by lymphocytes. In the spleens of some of the animals multinucleated cells occurred in or at the periphery of the tubercles. Lesions in the kidney were rare and when found consisted of periglomerular collections of monocytes.

Summary. *In vitro,* aureomycin compares

7. Steinbach, M. M., Duca, C. J., and Molomut, N., *Amer. Rev. Tuberc.,* 1944, v49, 105.

favorably with streptomycin and para-aminosalicylic acid in its antibiotic activity against *Mycobacterium tuberculosis* $H_{37}RV$.

In vivo, aureomycin proved ineffective as a therapeutic antibiotic agent in experimental tuberculosis of dba mice.

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Effect of Aminoguanidine on Formaldehyde Poisoning in Rats. (17987)

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Poisonings due to the handling of formaldehyde or the ingestion of methyl alcohol have been reported repeatedly(1). Treatment of such poisoning is symptomatic, including gastric lavage, administration of alkaline salts and morphine. The acidosis may be alleviated by intravenous infusion of glucose and sodium lactate. No specific antidote is known(2). Recently Bernheim(3) has shown that in the presence of aminoguanidine the oxidation rate of formaldehyde by rat liver is greatly increased. This suggested aminoguanidine as a possible antidote for formaldehyde poisoning. Its effect on methyl alcohol poisoning was also tested since some methyl alcohol is oxidized to formaldehyde which may contribute to its toxic effects(4).

Methods. White rats were injected intraperitoneally with minimum fatal doses of formaldehyde (180 mg/kg) and methyl alcohol (10 g/kg). Aminoguanidine sulfate, a relatively nontoxic substance, was injected in doses of 200-600 mg/kg. Since the doses of the toxic substances caused death in 5-30 minutes, it was necessary to inject the aminoguanidine shortly after the aldehyde or alcohol

TABLE I.
Effect of Aminoguanidine on Survival Time of Formaldehyde- and Methyl Alcohol-Poisoned Rats.

	No. animals used	% surviving at				
		30 min.	1 hr	8 hr	24 hr	4 days
Formaldehyde 180 mg/kg	15	0	—	—	—	—
Formaldehyde 180 mg/kg + Aminoguanidine 200 mg/kg	10	100	40	0	—	—
Formaldehyde 180 mg/kg + Aminoguanidine 400 mg/kg	20	100	100	80	60	20
Formaldehyde 180 mg/kg + Aminoguanidine 600 mg/kg	20	100	100	100	100	60
Methyl alcohol 10 mg/kg	10	0	—	—	—	—
Methyl alcohol 10 mg/kg + Aminoguanidine 600 mg/kg	15	0	—	—	—	—

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3. Bernheim, F., *J. Biol. Chem.*, in press.

4. Williams, R. T., Detoxication Mechanisms. Willy, New York, 1947.

had been introduced. In several experiments the aminoguanidine was injected first.

Results. Following injection of formaldehyde, 200 mg/kg aminoguanidine prolonged life slightly but afforded no significant protection. On the other hand 400 mg/kg of aminoguanidine prolonged the life of 60% of the animals more than 24 hours, 20% of the animals being alive after 4 days. When aminoguanidine was administered in doses of 600 mg/kg all animals were alive at the end of a 24-hour period, 60% surviving more than 4 days (Table I). Injection of aminoguanidine previous to the administration of formaldehyde raised these figures slightly. Repeated injection of aminoguanidine was without significant effect.

When methyl alcohol was administered aminoguanidine was without effect in the doses employed. This indicates that the acute toxicity of this substance is due to the depressant effect of the alcohol *per se*, particularly on respiration, and not to the accumulation of formaldehyde. It is possible, however, that some of the effects of chronic methyl alcohol poisoning could be prevented by aminoguanidine.

Summary. Aminoguanidine protects rats against the acute toxic effects of formaldehyde. Following methyl alcohol it is without effect, which indicates that formaldehyde does not contribute significantly to the acute toxicity of this substance.

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Development of Resistance to Folic Acid Antagonists in a Transplantable Lymphoid Leukemia. (17988)

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The development of resistance or insusceptibility to specific substances is a problem of much theoretical and practical interest. The production of resistant microorganisms *in vitro* and *in vivo* to various antimicrobial agents *viz.* antibiotics, antivitamins and bacteriophage has been recorded(1-6). The exact mechanism of this response is not known but has been postulated. It is possible that the development of resistance results from 1) emergence of resistant organisms due to selection, 2) action of the antimicrobial agent on the organism producing a variant, or 3), the development of gene mutations responsible for the resistance but having no direct relationship to the agent used. During the course of studies on the effect of several folic acid antagonists on transplantable acute lymphoid leukemias of the mouse, it has been noted that following a relatively long period of inhibition of leukemic cell growth, there resulted eventually a precipitous exacerbation of the disease with rapid escape of lymphoblasts into the blood, a generalized systemic infiltration into many organs and rapid growth of the localized lymphoma tumor mass in subcutaneously inoculated mice. Thus it was indicated that a resistance to the antifolic compounds had developed despite continued injections of the compounds at levels near the MTD. It was the purpose of this study to 1) attempt to induce resistance in mammalian leukemic cells to PGA antagonists, and 2) to study the characteristics of

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this resistance in the hope of establishing a rational approach to the chemotherapy of leukemia.

The experimental study reported herein relates to the response of 3 separate sublines of acute lymphoid leukemia, L1210(7) in consecutive transplant generations, to the MTD dosage of 4-amino-9-methyl PGA (A-ninopterin), 4 amino-9, N^{10}-dimethyl PGA (A-denopterin) and 4-amino-N^{10}-methyl PGA (A-methopterin) respectively.* Inoculations subcutaneously of leukemic cells into strain dba, subline 212, mice were done by use of a Lockes' solution suspension of spleen, lymph nodes and tumor mass as described previously(8). Beginning with the 60th transplant generation of leukemia L1210 (A-ninopterin and A-denopterin groups) and with the 65th transplant generation (A-methopterin group), the inoculated mice of each experimental series were divided into 2 groups, those treated with the folic acid antagonist and those continued untreated as controls. The estimated MTD for each antagonist, 3 mg/kg, was injected intraperitoneally (.01 cc/g body weight) every other day beginning at 48 hours after inoculation. Injections of the PGA antagonists were continued every other day until the subcutaneous growth of leukemic cells was large enough to transplant. Thus, in the early transfer generations the total dosage of antagonist given was in the order of 24 mg/kg. When resistance of the transplanted leukemic cells to the PGA antagonist developed, the total dosage given in each subline was 12 mg/kg body weight. Transfer of each subline, designated hereafter as AM-resistant, AD-resistant and AN-resistant, was continued through mice receiving the PGA antagonists. An attempt was made in all 3 sublines to transplant the leukemic cells as soon as possible when the growth was large enough to transplant as a brei suspension to at least 10 mice.

Burchenal *et al.*(9) reported the development of resistance of leukemia Ak-4 to repeated doses of 4-amino-N^{10}-methyl PGA using average survival time as the criterion. We have preferred to use a more exact measure of the growth of leukemic tissue and have used throughout the study reported here the mean weight in milligrams at 9 days of the localized lymphoma tissue, following subcutaneous inoculation.

In subline AN-resistant, given 4-amino-9-methyl PGA, no growth of the localized lymphoma mass at 9 days was observed during the first 4 transplant generations. The first palpable growth was found at 18 days in the first 3 transplant generations and at 10 days in the 4th transplant generation. At 9 days the mean weight of the tumor of the 5th transplant generation was 202.9 mg. This subline has now been carried through 13 transplant generations and continues to be resistant to the usual MTD of 3 mg/kg; the localized lymphoma cells growing profusely and attaining a size approximating that of the control line.

Subline AD-resistant treated with the MTD of 4 amino-9, N^{10}-dimethyl PGA remained sensitive to the antagonist through the first 4 transplant generations. At 9 days no growth of the tumor mass was observed; the first palpable growth was detected at 15 days in the first 2 transplant generations, and at 12 days in transplant generations 3 and 4. The mean weight of the lymphoma mass in the 5th generation was 104.9 mg. During the subsequent 10 transplant generations this subline has remained resistant to the usual MTD of 3 mg/kg of 4-amino-9, N^{10}-dimethyl PGA, attaining a mean weight of lymphoma tissue at 9 days of approximately 450 mg.

At the 9th transplant generation in treated mice an additional subline of the AD-resistant group was established by transplantation into dba mice which did not receive this PGA antagonist. This subline has now been carried through 7 generations of untreated mice and remains resistant to therapy with 3 mg/kg of 4-amino-9, N^{10}-dimethyl PGA.

A third subline, subline AM-resistant,

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transplanted through dba mice treated with the MTD, 3 mg/kg, of 4 amino-N^{10}-methyl PGA remained susceptible to the antagonist through 5 transplant generations. No growth was present during these passages at 9 days and the first palpable growths were found at 18, 18, 12, 14 and 22 days respectively. Complete resistance was evident in the 6th transplant generation. The mean weight of the lymphoma mass at 9 days was 741.3 mg. This subline has remained resistant, to the present time, through 12 transplant generations, with continued administration of 3 mg/kg of 4-amino-N^{10}-methyl PGA, attaining at 9 days growth comparable to the untreated control line.

Upon development of resistance in all 3 sublines investigated, a pronounced systemic infiltration into subcutaneous and internal lymph nodes, spleen, liver, thymus, kidneys and ovaries and escape of lymphoblasts into the blood stream was evident at 9 days. This is in contrast to the control line which showed no localized growth of lymphoma cells, slight if any systemic infiltration and very few lymphoblasts in the blood at 9 days following a total of 4 injections, every other day of the MTD of any of the 3 PGA antagonists investigated.

There were observed no histologic differences between the resistant and control susceptible tissues examined.

In contrast to the resistance to the PGA antagonists observed in these 3 transplant sublines, identical generations of control L1210 leukemic tissue carried in transplant in untreated strain dba mice have remained susceptible to the MTD of each of these PGA antagonists.

It was found that resistance to each of the antivitamins in the 3 transplant sublines of leukemia L1210 investigated was not independent of resistance to other closely related antagonists. For example, growth of leukemic cells of subline AD-resistant was not inhibited by injections every other day for 4 days of 3 mg/kg of 4-amino-9-methyl PGA nor of 4-amino-N^{10}-methyl PGA. Growth of leukemic cells of subline AM-resistant was not inhibited by the usual therapeutic dose, 20 mg/kg of 4-amino-pteroylaspartic acid, and growth of leukemic cells of subline AN-resistant following treatment with the MTD of 4-amino-9-N^{10}-dimethyl PGA (3 mg/kg) was comparable to the growth obtained following administration of 3 mg/kg of 4 amino-9-methyl PGA. Complete inhibition of the growth of leukemic cells at 9 days was obtained in the comparable transplant generations of the control leukemia in mice given the MTD of each of these antagonists. Determinations of the effects of other specific antileukemic agents on these resistant sublines are now under way.

Further studies with subline AM-resistant leukemia L1210 revealed that leukemic cells of this subline grew better in the presence of the antagonist, 4-amino-N^{10}-methyl PGA than in the absence of the compound. Pteroylglutamic acid had no apparent effect on the growth of leukemic cells of this subline. The growth obtained at 9 days, in this resistant subline, following intraperitoneal injections of 30 mg/kg body weight every other day for 4 days was essentially that of control (untreated) mice and approximately one-half the mean weight of growth in mice given injections of the PGA antagonist (Table I). Considerable variation in mean weights of the lymphoma tumor mass occurred among early transplant generations following the development of resistance indicating that a complete selection of resistant cells had not been attained. In the later transplant generations used variability in growth among the transplant generations was reduced.

A solution of the mechanism responsible for the development of resistance of mammalian cells reported here is probably not possible with the present material. A leukemic cell population originating from a single transplanted cell(10), however, would provide for a study of the mechanism involved. Such studies are now in progress.

Summary. A development of resistance has been shown in 3 separate sublines of a transplantable lymphoid leukemia, L1210, in strain dba mice following successive transplants in mice treated with the MTD of 3 different PGA antagonists: 4-amino-N^{10} methyl PGA,

10. Furth, J. and Kahn, M. C., *Am. J. Cancer,* 1937, v31, 276.

TABLE I.
Effect of 4-amino-N10 Methyl-pteroylglutamic Acid and Pteroylglutamic Acid on Subline AM-resistant Lymphoid Leukemia L1210.

Experiment	Daily dosage, mg/kg	Transplant generation	No. of mice	Mean wt of tumor, mg*	Absolute No. lymphoblasts (peripheral blood)
AM-Resistant L1210					
4-amino-N10-methyl-pteroylglutamic acid	3.0		38	388.5	1430†
Pteroylglutamic acid	30	G7 to G11	34	159.0 } 176.7	—
Controls	—		20	207.7 }	—
Diff. of means = 211.8 ± 54.6 mg; t = 3.9; P >0.01‡					
4 amino-N10-methyl-pteroylglutamic acid	2.5	G12	12	750.2	
Controls	—	,,	13	336.5	
Diff. of means = 413.7 ± 59.2 mg; t = 6.9; P >0.001					
Control L1210					
4 amino-N10-methyl-pteroylglutamic acid	3.0	G1 to G6	25	No growth	
Pteroylglutamic acid	30	G6	5	544.0	
Controls	—		5	494.3	
4 amino-N10-methyl-pteroylglutamic acid	2.5		5	28.6	300
Pteroylglutamic acid	30	G12	5	581.7	—
Controls	—		5	808.1	1750

* Wet wt obtained on a Roller-Smith micro torsion balance.

† The mean absolute number obtained from 6 mice, transplant generations 6, 7, and 8.

‡ Since the difference of the mean weights of the tumor mass in the pteroylglutamic acid group and the control group was not significant statistically, the mean weight of pooled data for these 2 groups (176.7) mg is compared with the mean weight of the 4-amino-N10-methyl-pteroylglutamic acid group.

4-amino-9, N^{10} dimethyl PGA and 4-amino-9 methyl PGA. All 3 sublines continue to be resistant in subsequent transplant generations following emergence of resistance. Subline AD-resistant leukemic cells grown in mice not given the folic acid antagonist for 7 successive transplant generations continue to be resistant to 4-amino-9, N^{10} dimethyl PGA. Resistance developed to one of these antivitamins was found not to be independent of resistance to the others. AM-resistant leukemic cells were found to grow better in animals receiving the MTD of 4-amino-N^{10} methyl PGA than in animals untreated with this antivitamin or given PGA parenterally.

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Diabetic State with Lipaemia and Hydropic Changes in the Pancreas Produced in Rabbits by Cortisone.* (17989)

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During the course of an experiment undertaken with the purpose of observing the effect of Cortisone on the tissue lesions produced by foreign serum proteins in rabbits living in a cold environment, a marked alteration of carbohydrate metabolism which resembled a diabetic state and abnormalities of the Islets of

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† This work was done during the tenure of a Life Insurance Medical Research Fellowship.

TABLE I.
Biochemical Findings in the Blood and Serum of Two Rabbits Treated with Cortisone in a Dose of 20 mg Per Day.

Rabbit No.	Day of cortisone	Blood sugar, mg %	Visible lipaemia	Lipid phosphorus, mg %	Cholesterol, mg %		Fatty acids of neutral fat, m.Eq./liter
					Free	Total	
K-33	2	180	0				
	5	203	++				
	8	310	++	25.4	65.0	90.0	43.99
	12	688	++++	30.6	50.0	250.0	69.57
K-37	—1	133	0				
	2	209	0				
	5	272	+	14.8	36.0	50.0	23.55
	8	367	++	29.0	100.0	330.0	57.23
	15	448	+++				
	22	1280	++++	61.2	425.0	500.0	742.8
Normal rabbit serum	Payne & Duff (2)	42 rabbits		4.4 S.D.* 1.7	19 S.D. 9.5	46 S.D. 17.5	7.3 S.D. 3.1

* S.D. = Standard deviation.

Langerhans were observed. These were accompanied by a profound effect on lipid metabolism. Since these changes did not occur in control animals kept under the same conditions but not given Cortisone, the abnormalities are attributable to the Cortisone. So far as we are aware, the occurrence of lipaemia and lesions of the islets have not heretofore been reported as resulting from the treatment of animals with Cortisone, although McLean(1) has observed the occurrence of visible lipaemia in rabbits treated with Cortisone and horse serum.

After a suitable period of observation during which time it was established that the animals did not manifest a significant glycosuria, albuminuria, elevation of the blood sugar or visible lipaemia, 2 rabbits were treated with 2 intramuscular injections of 10 mg of Cortisone acetate (Merck) each day. On the third day of treatment with Cortisone, the animals were placed in a cold room in which the temperature varied between 8 and 30°F, remaining near the lower limit for most of the day. On the sixth day, each animal received an intravenous injection of 10 ml of normal horse serum per kilo of body weight. One animal (K-33) died on the 12th day and the other (K-37) was killed on the 22nd day after the commencement of the Cortisone treatment. Pairs of controls receiving no treatment or intravenous horse serum only, were kept in the cold room at the same time. Food and water were given *ad libitum.* Though the conditions of the experiment are detailed, it appears from our observations on animals presently under study, that the lipaemia, hyperglycemia and glycosuria appear even if the animals are kept at ordinary room temperature and no serum is administered.

Five days after the commencement of Cortisone, a definite cloudiness of the serum was evident to the naked eye. On chemical analysis, the serum manifested an increase in all of the lipid fractions which was similar in pattern to that reported from this laboratory by Payne and Duff(2) in alloxan diabetes in the rabbit, but reaching somewhat higher values. The findings in the serum are detailed in Table I. The blood sugar was markedly elevated and the rise in the lipid content of the serum paralleled the rise in the blood sugar (Table I). A marked glycosuria occurred corresponding to the hyperglycemia.

The islets of the animal that died on the 12th day of the treatment (K-33), showed some swelling of the cells with an occasional one showing fragmentation of the cell border.

1. McLean, C. R., unpublished observations.

2. Payne, T. P. B., and Duff, G. L., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 332.

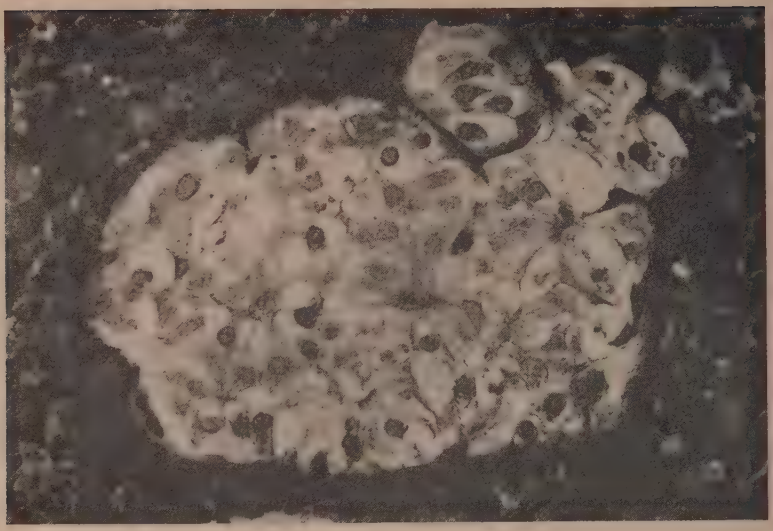

FIG. 1.
Islet of Langerhans from animal K-37 showing marked "hydropic change" of the beta cells. Iron haematoxylin, phloxine × 400.

It is possible that these changes were due to postmortem autolysis, since the animal died and was autopsied 18 hours after death. However, autolysis does not usually occur if animals die in the cold room, even if 24 hours elapse between death and autopsy. The animal that was killed on the 22nd day (K-37) and autopsied immediately, showed a marked vacuolation or "hydropic change" of the cells of the islets and ductules of the pancreas (Fig. 1). As first demonstrated by Toreson (3) in this laboratory in experimental diabetes produced by anterior pituitary extract, partial pancreatectomy or alloxan, and in human diabetes, the so-called hydropic cells contained glycogen stainable with Best's carmine, but not stainable in diastase-treated control sections. Gomori's stain showed that the vacuolation was chiefly in the beta cells sparing the alpha cells.

The full significance of the observations cannot be appreciated at this time, since they are not complete. However, it appears that a state very similar to a true diabetic state has been produced in rabbits with Cortisone. This state is accompanied by a lesion of the islets similar to that seen in other forms of diabetes, both experimental and human(3). Whether the lipaemia is a derangement of lipid metabolism accounted for by the direct effect of Cortisone, or whether it is secondary to the disturbance of carbohydrate metabolism and the diabetic state cannot be decided from consideration of the data at hand.

The fact that the metabolic disturbances here reported occur in rabbits under Cortisone therapy provides another possible experimental approach to the study of the diabetic state. The occurrence of lipaemia in association with a disturbance of carbohydrate metabolism simulating diabetes suggests that Cortisone may have similar effects on experimental atherosclerosis to those recently observed by Duff and McMillan(4) who demonstrated that alloxan diabetes has an inhibitory effect on cholesterol arteriosclerosis. Duff and Payne(5) have since shown that this effect is correlated with a marked elevation of the

3. Toreson, W. E., Abstract, *Am. J. Path.*, 1950, v26, in press.

4. Duff, G. L., and McMillan, G. C., *Exp. Med.*, 1949, v89, 611.

5. Duff, G. L., and Payne, T. P. B., Abstract, *Am. Heart J.*, 1949, v38, 455.

neutral fats and phospholipid fractions in the serum.

The appearance of these deleterious effects in rabbits would seem to warrant a search for similar effects in other animal species including man.

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Alterations in Rate of Influenza Virus Proliferation Produced by Growth Hormone and Testosterone.* (17990)

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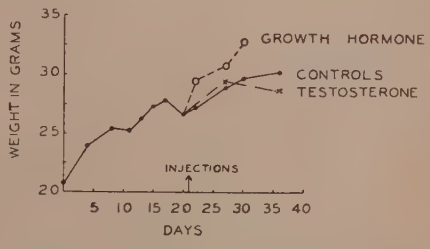
Recent studies have revealed marked differences in the susceptibility of mice of different ages to influenza virus infection. With advancing age there is a progressive increase in the amount of virus necessary to produce a lethal infection(1). These observations led to the development of the hypothesis that the rate of virus proliferation is, in part, dependent upon the rate of protein anabolism in the tissues of the host. It was, therefore, decided to study the effect on virus proliferation of deliberate alterations in the protein anabolic activity of the host. Since it is well established that the administration of pituitary growth hormone and testosterone results in increased rates of protein synthesis in the host(2,3), these substances were selected for use. The following report, then, is concerned with the rates of virus proliferation in growth hormone-treated and testosterone-treated mice as compared with appropriate controls.

Materials and methods. Female Swiss mice were employed throughout these experiments. They were obtained from the same source at the age of approximately 8 weeks and maintained in the laboratory on Purina dog chow and water *ad libitum*. The mice were weighed 3 times weekly, and, after a period of about 20 days, their weight curves "plateaued."

Both hormones were administered by subcutaneous injection. The growth hormone‡ was given for a period of 10 days, each mouse receiving 0.2 mg in a single injection per day. Testosterone‡ was given in 3 weekly injections, each mouse receiving 5.0 mg per week of a commercially prepared aqueous suspension. Control mice received appropriate injections of the vehicle in which the hormone was dissolved. The day after the last hormone injection, approximately 35-40 mice of both treated and control groups were inoculated intranasally with a 10^{-5} dilution of mouse-adapted PR 8 influenza virus. This inoculum was equivalent to approximately



THE EFFECT OF GROWTH HORMONE AND TESTOSTERONE ON THE WEIGHT OF MATURE FEMALE MICE.

FIG. 1.

* Aided by a grant from the Hendricks Research Fund.

† Submitted in partial fulfillment of the requirements for the degree of Master of Science.

1. Kalter, S. S., *J. Immunol.*, 1949, v63, 17.

2. Li, C. H., *Growth*, 1948, v12 suppl., 47.

3. Kochakian, C. D., In R. S. Harris and K. V. Thimann, Vitamins and Hormones, 1946, v4, 255.

‡ We wish to thank Irby Bunding of Armour and Co. for a generous supply of Growth Hormone (Lot 22kR1) and Fred Houghton of Ciba Pharmaceutical Products, for the Testosterone used in this study.

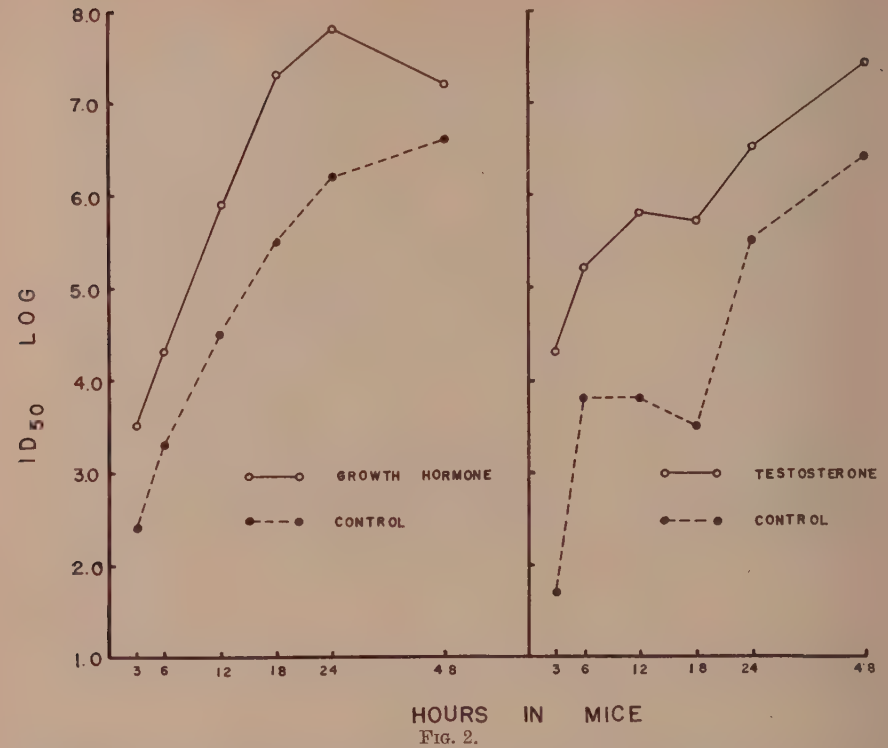


FIG. 2.

The effect of growth hormone and testosterone on the rate of proliferation and amount of influenza virus in mice. The ID_{50} was determined by titration in chick embryos.

500 x the LD_{50} dose when titrated in 4-5-week-old mice. Following virus inoculation, 5 treated and 5 control mice were killed, the lungs removed and blotted on filter paper, pooled in their respective groups, weighed, quickly frozen and stored at -70°C. The mice were sacrificed 3, 6, 12, 18, 24 and 48 hours after infection. When all the samples had been collected, 10% suspensions of the lungs were homogenized with buffered saline in a Waring blendor and centrifuged. For the egg titrations, dilutions were made in buffered saline, containing sufficient penicillin and streptomycin to insure sterility, and each dilution inoculated into 11-day-old fertile embryos. Five embryos were employed for each dilution. The allantoic fluids were harvested in the usual manner and the LD_{50} determined by the method of Reed and Muench. Hemagglutination tests were also made of the mouse lung preparations in the usual manner(4).

Results. The effect of growth hormone and testosterone on the weights of the mice may be seen in Fig. 1. Approximately a 4.0 g increase in weight was obtained by using the growth hormone. No significant weight difference was observed following the use of the testosterone.

Treatment with both hormones resulted in an increased rate of virus proliferation and an increase in amount of virus present. This is demonstrated in Fig. 2. Little difference in virus proliferation was apparent between the growth hormone and testosterone treated animals. These experiments were repeated and the same relationship was observed between the treated and control animals.

4. Hirst, G. K., *Science*, 1941, v94, 22.

Hemagglutination determinations of the lung suspensions also demonstrated a difference between the treated and control animals. However, this method of assay which is known to be less sensitive than the *in ovo* titration, revealed less striking differences between experimental and control groups.

Discussion and conclusions. If virus proliferation is partially dependent upon the host's protein synthetic mechanisms, an increase in the rate of protein anabolism in the host may be reflected in an increased rate of virus growth. Under the conditions of these experiments, the data obtained are not inconsistent with this hypothesis.

Summary. When the rate of protein anabolism in the host was increased by testosterone or growth hormone, increases in rate of proliferation and amount of influenza virus were clearly demonstrated.

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A Rapid Method for Detection of Influenza Virus During Epidemics.* (17991)

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The established procedures for the detection of influenza in a community are: (a) isolation of the virus, (b) serological examination of acute and convalescent phase serum specimens, and (c) the inoculation of susceptible animals with throat washings. Regardless of the method, these procedures require several days as a minimum before identification of the agent may be made. During the current epidemic, a screening method has been employed which enables the laboratory to ascertain the causative agent in one day.

Materials and methods. Twenty-eight throat washings were obtained during the epidemic from patients in the student infirmary. These patients were admitted with the clinical diagnosis of either influenza or respiratory infection. Usually the throat washing was obtained during the first 3 days of illness. The patients first coughed several times and then gargled with approximately 15 ml of nutrient broth. These throat washings were then quickly frozen and stored at —70°C until tested. Control throat washings were obtained from 10 persons who gave no history of illness during the previous 3 week period. The throat washings from patients and controls were treated in an identical manner.

The throat washings were thawed under running water and 10 ml removed. The remaining 5 ml was used for isolation of virus by amniotic inoculation of 13-day-old chick embryos. Sufficient 1% red blood cells (either chicken or human type O) in buffered saline were added to each throat washing in order to compensate for the hemolysis which sometimes occurred. This was usually in the order of 2-4 ml. After standing at room temperature for 1¼ hours, the material was centrifuged and the supernatant fluids discarded. To the red cell sediment was added 0.8 ml saline, the mixture shaken and incubated in a 37°C water bath for 2-4 hours with occasional shaking. Following this period of elution, the material was again centrifuged and the supernatant fluids removed to small tubes. Then 0.6 ml of each supernatant was removed and 0.2 ml placed in each of 3 tubes. To the first tube, 0.2 ml 1% fresh erythrocytes were added, to each of the other 0.2 ml samples, 0.2 ml red cells (1%) in a 1:50 dilution of specific antiserum was added. The antisera employed were PR 8, Lee and FM-1. The FM-1 antiserum was

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TABLE I.
The Determination of Influenza Virus in Throat Washings by Absorption and Elution from Red Blood Cells.

No. of throat washings	Positive agg. after absorption	Positive agg. after elution	Specific neutralization		
			PR 8	Lee	FM-1
28	12	8*	0	0	5
Controls					
10	1	0	0	0	0

* Three of these preparations were not inhibited by any of the antisera.

obtained through the courtesy of Dr. Irving Gordon, Division of Laboratories and Research, Albany, N. Y. These hemagglutination tests were read after 30 minutes and 1¼ hours in the usual manner. Care must be exerted in the reading of the tests as the amount of virus present is small. The results are more in the order of a weak positive, the complete agglutination seen with allantoic fluids containing virus is rare. Readings after 30 minutes are helpful inasmuch as there is a difference in the rate of settling of the cells. A few preliminary experiments with 0.5% red cell suspensions indicate a sharper end point.

Results. As seen in Table I, positive identification of the causative agent was obtained in 5 throat washings of the 28 tested. No positive agglutinations were obtained with the control throat washings. Although isolation of influenza virus was not attempted with all 28 specimens, the virus was isolated and identified as a strain resembling FM-1. Isolation of virus was obtained from 3 of the preparations which showed specific neutralization, the other 2 were not tested. From 2 throat washings which did not react to this method, virus was also isolated. From one preparation in which hemagglutination occurred after elution no virus was isolated. No attempts were made to isolate virus from the control preparations.

With some throat washings, 0.5 ml saline was used for elution. In these cases, insufficient material was available for specific identification. Therefore, a multi-valent antiserum (from patients with high titers to the three strains of virus) was employed. Agglutination inhibition was obtained in some of these preparations. The results of complement-fixation tests with sera from these patients indicated the presence of an influenza virus closely related to FM-1.

Discussion. This procedure appears to be of value as a screening method during epidemics of influenza. When large numbers of throat washings are available, random samples may be screened by this procedure in order to identify the strain of influenza virus present in the community. Confirmation using established procedures can then be made. The results obtained by this rapid method were confirmed by established procedures and no false positives were obtained from control material. It is obvious that this procedure is only valuable when a large number of specimens are available. A certain number of specimens containing small amounts of virus will yield negative results by this method and positive results by the standard methods.

It is possible that a modification of the technic may be of value in increasing the concentration of influenza virus in order to enhance isolation procedures. It may also be possible to utilize this method for the isolation of other viruses such as mumps.

Non specific positives are obtained in certain specimens which may be due to the activity of saliva(1) or other agglutinating agents.

Summary. A rapid method for the detection of influenza virus in throat washings during epidemics is described and discussed. Essentially this method consists of concentrating and eluting the virus by red cell adsorption and then neutralizing with specific antisera.

1. Francis, T., Jr., and Minuse, E., Proc. Soc. Exp. Biol. and Med., 1948, v69, 291.

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Influence of Sex Hormones on Tolerance to Aminopterin.* (17992)

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(Introduced by A. Mazur.)

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Folic acid as a dietary essential for proper biological activity of estrogen has been demonstrated by the reduction of feathering(1) and oviduct response(2) in stilbestrol-treated chicks, absence of normal estrogen response in immature monkeys(3) and ovariectomized rats(4) on folic acid deficient diets. Impairment of reproductive performance of rats(5) results from a pregestational folic acid deficient diet which is further accentuated by the addition of an antifolic acid chemical. The folic acid antagonist, aminopterin (4-amino-pteroylglutamic acid) used to induce a deficiency, decreases(6) the oviduct response to estrogen in newly metamorphosed frogs and interferes(7) with the depressive influence of estradiol on the rat prostate. A quantitative relationship exists between folic acid and estrogen(2); the frog's oviduct response is increased by folic acid(6). Aminopterin possesses no androgenic activity(7) itself and does not interfere with androgen stimulation. However, a folic acid deficiency produces a more effective comb growth response to androsterone in chicks(4).

Folic acid, therefore, might be instrumental in inhibiting testosterone induced tissue hypertrophy and a deficiency may be the instrument for inhibition of estrogen induced tissue hypertrophy. Since a quantitative relationship between folic acid and estrogen has been established, indicating a higher estrogen response with greater folic acid intake(2), it might be conceivable that the folic acid requirement is increased in estrogen administration. Hertz(2) has pointed out the destruction of bone marrow by prolonged administration of stilbestrol, which might represent an increased folic acid requirement for bone marrow protection. If the sex hormones influence the folic acid requirement of the body, the measurement of tolerance to an induced folic acid deficiency in the presence and absence of these hormones should demonstrate an appreciable difference. Tolerance to aminopterin, a competitive vitamer, serves as a means of quantitatively determining this factor.

Procedure. Toxicity studies of aminopterin‡ were conducted on male and female albino (Swiss strain) mice, in 4 groups of experimental animals: (1) mature; (2) mature hormone treated; (3) gonadectomized; (4) immature. Two series were conducted on the mature mice in order to establish the more favorable synthetic folic acid deficient diet for this study. Mature mice in series A were fed, *ad libitum,* the diet 566 of Spicer *et al.* (8) in which intestinal synthesis of folic acid was not inhibited. Mature mice in series B were fed the folic acid deficient diet of Franklin *et al.*(9) in which intestinal synthesis of folic acid was eliminated by the addition of

* Aided by the United States Public Health Service, Research Grant No. C-629 (C).

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2. Hertz, R., *Endocr.,* 1945, v37, 1.

3. Hertz, R., Proc. Soc. Exp. Biol. and Med., 1944, v67, 113.

4. Hertz, R., Recent Progress in Hormone Research, II, Academic Press, 1948, 161.

5. Nelson, M. M., and Evans, H. M., *J. Nutr.,* 1949, v38, 11.

6. Goldsmith, E. D., Schreiber, S. S., and Nigrelli, R. F., Proc. Soc. Exp. Biol. and Med., 1948, v69, 299.

7. Brendler, H., *Science,* 1949, v110, 119.

‡ Furnished through the courtesy of Lederle Laboratories Division, American Cyanamid Company, Pearl River, N. Y.

8. Spicer, S. S., Daft, F., Sebrell, W. H., and Ashburn, L. L., *U. S. Pub. Health Rep.,* 1942, v57, 1559.

9. Franklin, A. L., Stokstad, E. L. R., and Jukes, T. H., Proc. Soc. Exp. Biol. and Med., 1947, v65, 368.

TABLE I.
Comparison of Aminopterin Mean Lethal Doses in γ/g in Male and Female Albino Mice.

Mice	Males		Females		Mean diff.	Stand. error diff.	Ratio mean diff. to stand. error diff.
	Mean	± std. dev.	Mean	± std. dev.			
Mature A*	1.62	.63	2.58	2.48	.96	.17	6.75 significant
B	1.31	.50	1.63	.67	.36	.13	2.46 "
Mature hormone treated†	1.46	.18	1.81	.17	.35	.15	2.38 "
Gonadectomized	1.55	.24	1.58	.8	.03	.16	.15 not "
Immature	1.54	.42	1.7	.2	.16	.13	1.2 " "

* Series A of mature mice on folic acid deficient diet 566 of Spicer *et al.*(8), intestinal synthesis of folic acid permitted. All other animals were on folic acid deficient diet of Franklin *et al.*(9), intestinal synthesis of folic acid inhibited.

† Males received 5 γ estradiol benzoate injections subcutaneously twice weekly. Females received 50 γ testosterone propionate injections subcutaneously twice weekly.

1% succinyl sulfathiazole to the diet. All animals in other experimental groups received this diet of Franklin *et al.*(9). There were two groups of control animals of both sexes. One group received the folic acid deficient diet(9) and the other a diet made more complete by the addition of folic acid and the exclusion of sulfathiazole(9). The general procedure for all four of the following experimental groups was the same. Mice were weighed at the beginning of the experiment and a periodic checking of weights was made throughout the course of the experiment. Saline solution of aminopterin was administered intraperitoneally daily and the lethal doses were calculated in γ/g on the basis of the total toxic dose and the initial weight.

1. *Mature mice.* Series A consisted of 50 mature males and females which received daily doses of aminopterin varying from .5 to 3γ. Series B consisted of 64 mature males and females which were given doses of 1 to 3γ aminopterin daily.

2. *Mature hormone treated mice.* A group of 64 mature hormone treated males and females were given daily aminopterin doses of 2 and 3γ. Subcutaneous injections of 5γ estradiol benzoate§ to males and 50γ testosterone propionate§ to females were administered twice weekly. This quantity of estradiol is sufficient to produce a full estrogenic smear in ovariectomized females and the quantity of testosterone produces a 100% increase in the size of the seminal vesicle.

3. *Gonadectomized mice.* A group of 54 males and females (2-3 months old) gonadectomized at 3 weeks of age, received daily aminopterin doses of 2 and 3γ.

4. *Immature mice.* Fifty male and female weanling mice received daily aminopterin injections of 3γ.

Results. 1. *Mature mice.* In Series A on mature mice, in which intestinal synthesis of folic acid was not inhibited, the range in lethal dose was very wide and varied from .4 to 8.8 γ/g. However, a marked sex difference in tolerance to aminopterin was apparent (Table I) when the mean lethal doses were compared. The difference between the 2.58 for females and the 1.62 for males was very significant and indicated the greater toxicity of aminopterin in males. The intestinal synthesis of folic acid accounted for the large distribution in lethal dose. The ability of some mice to tolerate considerably higher doses was attributed to the variation in the amount of folic acid synthesis. When the mean lethal doses of Series A and B on mature mice were compared, it was demonstrated that inability to synthesize folic acid produced less individual variation as well as lowered tolerance (Table II). The range for Series B was not as great and varied from .5

§ The estradiol benzoate and testosterone propionate were supplied by Ciba Pharmaceutical Products, Inc.

TABLE II.
Comparison of Aminopterin Mean Lethal Doses in γ/g in Mature Albino Mice on Different Diets

	Males			Females		
Mature mice compared	Mean diff.	Stand. error diff.	Ratio mean diff. to stand. error diff.	Mean diff.	Stand. error diff.	Ratio mean diff. to stand. error diff.
Series A,* B†	.95	.43	2.2 significant	.31	.18	1.78 significant

* Series A on folic acid deficient diet 566 of Spicer *et al.*(8), intestinal synthesis of folic acid permitted.

† Series B on folic acid deficient diet of Franklin *et al.*(9), intestinal synthesis of folic acid inhibited.

TABLE III.
Comparison of Aminopterin Mean Lethal Doses in γ/g in Various Experimental Groups of Albino Mice on the Same Diet.*

	Males			Females		
Mice compared	Mean diff.	Stand. error diff.	Ratio mean diff. to stand. error diff.	Mean diff.	Stand. error diff.	Ratio mean diff. to stand. error diff.
Mature (B) with gonadectomized	.24	.10	2.1 significant	.05	.20	.26 not significant
Mature (B) with immature	.23	.13	1.87 "	.07	.14	.48 " "
Mature (B) with hormone treated	.15	.14	1.07 not "	.18	.12	1.21 " "
Immature with gonadectomized	.01	.06	.17 not "	.13	.14	.88 " "

* Folic acid deficient diet of Franklin *et al.*(9), intestinal synthesis of folic acid inhibited.

to 3.1 γ/g. The mean lethal dose of 1.63 for females was significantly higher than the 1.31 for males (Table I). Schoenbach *et al.*(10) observed the same lethal dose in males. A lower tolerance of males to aminopterin was apparent in mice on both diets and the ability to synthesize folic acid increased tolerance of both males and females, though still maintaining a sex difference (Tables I and II).

2. *Mature hormone treated mice.* No influence of testosterone on the tolerance of mature females or estradiol on tolerance of mature males was demonstrated since the same statistically significant sex difference existed (Table I) as shown by a mean difference of .35 γ. In addition there were no significant differences between the mean lethal doses of this group and the untreated mature mice (Table III).

3. *Gonadectomized mice.* Gonadectomy increased the mean lethal dose of males (Table I) to that of normal mature females. The mean lethal doses of gonadectomized males and females were equivalent (Table I) and are 1.55 and 1.58 γ/g respectively. Ovariectomy did not influence the tolerance and was demonstrated by no significant difference in mean lethal doses (Table III) of gonadectomized and normal mature female mice. Castration, however, had a marked effect on the lower male tolerance observed in mature mice and the mean lethal dose was increased by .24 γ (Table III).

4. *Immature mice.* No sex difference in aminopterin tolerance was manifested in immature mice as was similarly demonstrated in

10. Schoenbach, M. D., Goldin, A., Goldberg, B., and Ortega, L. G., *Cancer*, 1949, v2, 57.

gonadectomized mice (Table I). The mean lethal doses of weanlings did not differ significantly from those of gonadectomized mice (Table III).

In all experimental groups, signs of toxicity were apparent. Ruffed hair, bloody diarrhea and weight loss were noticeable. The control animals on folic acid deficient diet long outlived the experimental ones. The average length of survival of mice receiving aminopterin was one month. At the end of 6 months, 6 of the control mice were still living. Two of the males had died after 4 and 5 months.

Discussion. The toxicity of aminopterin in mice varies with diet and sex. When mice were capable of intestinal synthesis of folic acid, even as a sole source of supply, the tolerance to aminopterin was considerably increased. Since there was considerable variation in tolerance under these circumstances the mice were fed a more completely folic acid deficient diet by the addition of 1% succinyl sulfathiazole in all subsequent experiments. The sex difference in tolerance observed was attributed to testosterone which must exert a synergistic action with aminopterin. All mice devoid of testosterone (mature, immature and gonadectomized females and immature and gonadectomized males) demonstrated the same tolerance to aminopterin as indicated in similarity of mean lethal dose. Aminopterin was more toxic in mature males since the mean lethal dose was significantly lower than that of the testosterone poor group. Estrogen did not influence the folic acid requirement of the body nor did it exert any effect upon tolerance to aminopterin since its presence in mature females produced no change in mean lethal dose. Though an aminopterin induced folic acid deficiency decreases estrogen response(4,7), the effect of this deficiency must be directly on the end organ.

Testosterone itself is not toxic. However, its presence enhanced the toxic effect of an anti-folic drug such as aminopterin. It is possible that the folic acid requirement of the body was increased in the presence of testosterone. It may be that the mode of action of aminopterin was upon an end organ which was influenced by testosterone and synergism was thereby produced. In mature mice treated with hormones, testosterone was always present with estrogen. However, when administered to females, testosterone did not produce the synergism found in normal mature males even though the quantity would have been enough to increase the seminal vesicle size by 100%. Estrogen, in an amount high enough to produce a full estrogenic smear in gonadectomized females, did not mask the toxic effect of aminopterin in the male and had no influence on whatever end organ aminopterin affected. Mice with and without estrogen tolerated the same lethal dose. For further clarification, gonadectomized mice of both sexes should be given larger doses of testosterone. A specificity of the synergism between testosterone and aminopterin may exist or the action exerts its influence on any induced folic acid deficiency. Other anti-folic drugs should be investigated in the same manner.

Conclusion. Folic acid synthesis by intestinal flora decreased the toxicity of aminopterin in mice on an otherwise folic acid deficient diet. The addition of 1% succinyl sulfathiazole to the diet gave more consistent results though aminopterin tolerance was lowered. A lowered tolerance was observed in mature males which is attributed to a synergism between testosterone and aminopterin. A higher mean lethal dose was observed in all mice which did not have testosterone (mature, gonadectomized, immature females and gonadectomized, immature males). Estrogen had no influence on aminopterin tolerance.

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Inositol and the Toxicity of Four Isomers of Benzenehexachloride for the Rat. (17993)

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The widespread distribution of i-inositol and its unknown roles in the maintenance of life processes have stimulated search for an antimetabolite which might elucidate these problems. Microbiological(1,2), toxicological(3-6), and physical data(7,8) indicate that γ-benzenehexachloride (γ-BHC) is not the structural analogue of i-inositol, as was originally proposed by Slade(9). Consequently, examination of the other available isomers of benzenehexachloride for toxicity and anti-inositol properties was undertaken.

Technics and the basal diet* were identical with those employed previously(6). Each isomer† was incorporated in the diet at the level of 0.6 mg/g food by solution in lard, while i-inositol replaced an equivalent weight of sucrose. Twenty weanling 20-25 day-old male rats weighing 44-50 g each were placed on each diet, fed *ad libitum*, and weighed twice weekly for 4 weeks. The variables in the diets, growth rates and mortality are presented in Table I.

Comparison of growth rates of animals on the basal diet with and without added inositol indicates that this strain of rat requires no dietary inositol under these experimental conditions. α- and δ-BHC produced only minimal growth depression without fatality at the level fed. However, the β and γ isomers were relatively toxic. Significant growth retardation occurred only in animals receiving the γ isomer. At the level fed, this isomer produced an initial weight loss, growth retardation, characteristic irritability, hyperactivity, violent convulsions, and death in over 50% of the animals. This syndrome appeared in some animals within the first 24 hours of the experiment; of the 40 rats receiving γ-BHC, 20 were dead by the eleventh day. Tolerance to the drug then developed and near normal growth of survivors resulted. Neither the convulsive disorder nor the growth retardation was alleviated by i-inositol, fed at a ratio of 1:0.75 moles of γ-BHC. Other ratios up to 5.3:1.0 have produced similar results in the rat(6) and the mouse (unpublished data from this laboratory).

The β-isomer, on the other hand, produced no growth retardation during the first 2 weeks; the first signs of its toxicity, appearing about the seventh day, were increased docility, sluggish movements and torpor, followed infrequently by paralysis of the hind quarters. A progressive increase of lethargy to coma in all animals, interrupted occasionally by localized or generalized convulsions, with incontinence of urine and rare opisthotonus, was observed. After the development of narcosis, it was difficult to determine if the animals were dead or in antemortem coma, due to a progressive hypopnea and falling pulse rate for 48 hours preceding death. At this stage, a rapid loss of weight ensued. Of the 40 rats receiving the β-isomer, half were

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7. Bijvoet, J. M., *Rec. trav. chim.*, 1948, v67, 777.
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9. Slade, R. E., *Chem. and Ind.*, 1945, v64, 314.

* Basal diet: Labco casein, 15.0; l-cystine, 0.3; salts 3A, 2.0; salts 3B, 2.0; celluration, 2.0; B-complex mixture, 1.0; choline chloride, 0.4; fat soluble vitamin mixture, 2.0; lard, 8.0; sucrose, 67.3%.

† The authors with to express their thanks to Mr. Jerome Martin of Commercial Solvents Corporation for his generous gift of the 4 isomers, the uncorrected melting points were: α = 156.5-157.1; β = 311.5-312.0; γ = 112.0-113.0; δ = 136.5-137.0.

TABLE I.
Growth Rates and Mortality of Rats Receiving Isomers of BHC. (20 rats on each diet at start of experiment).

Basal diet supplement				Growth increments (g/wk)				
Inositol mg/g food	Isomer 0.6 mg/g food	Initial wt (avg) g	Final (28 d) wt, g	1	2	3	4	Avg g gain per wk
—	—*	45.7	177.3	27.7	32.3	36.4	35.2	32.9
.5	—	46.0	177.4	30.4	29.5	35.4	36.1	32.9
—	α*	46.8	168.7	24.5	31.2	32.9	33.3	30.5
.5	α*	46.6	171.5	25.8	34.7	34.0	30.4	30.5
—	β†	46.0	—	27.3	31.3	0		29.3‡
				(2)	(7)	(20)		
.5	β	46.7	—	28.3	33.1	0		30.7‡
				(1)	(8)	(20)		
—	γ	45.6	138.6	3.8	26.2	34.7	28.3	23.3
				(7)	(10)	(11)	(11)	
.5	γ	45.9	131.7	1.6	21.6	33.2	29.4	21.5
				(7)	(11)	(12)	(12)	
—	δ	45.7	165.5	19.8	32.4	34.6	33.0	30.0
.5	δ	45.5	167.4	22.1	33.5	33.3	33.0	30.5

* One rat died of an apparently pre-existing disease.
† Numbers in parentheses indicate total number of deaths.
‡ Avg gain over the 2 wk preceding death.

dead by the seventeenth day, the remainder dying before the twenty-third day. On post-mortem examination, the disagreeable odor accompanying benzenehexachloride was emitted, particularly from the abdominal organs. Aside from an empty gastrointestinal tract, absence of depot fat, minimal color changes in the kidneys and liver, and adrenal enlargement (possibly subsequent to coma-induced inanition), the only gross finding of interest was marked distension of the urinary bladder. The latter finding with the paresis noted previously resembled "cord bladder," suggesting a peripheral as well as central action of the β-isomer.

Microscopic examination of the tissues failed to reveal any significant findings. Tissues were immediately fixed in 4% formalin and stained with haematoxylin-eosin. The brain and spinal cord were examined in addition after Weigert's stain, osmic acid preparation, phosphotungstic acid, and Mallory's connective tissue stains. No gliosis, demyelinization or fat granulation were observed. In the β-BHC animals, a patchy non-specific pyknotic shrinkage of a few of the cortical ganglion cells, interpreted as a late phenomenon of profound coma was occasionally observed. No other changes in the neurons or neurocytes were evident. These observations suggest a biochemical rather than an anatomic lesion.

Food consumption was similar for the controls and the α, β, and δ-BHC groups, while the animals receiving γ isomer ate less. The most striking differences in food consumption are found in the first week, during which the γ-BHC groups consumed about half as much food as the other groups. Intakes were slightly depressed in the β and δ-BHC animals in the first week, returning to normal in the second week. The onset of fatal coma in the remaining β-BHC animals in the third week reduced their intake to zero in the fourth week.

The food intake of the animals determines the dosage of the isomer. The intakes of the

TABLE II.
Food Intake of Rats Receiving 0.6 mg BHC/g Food (g food/rat/wk).

Supplement	Wk of exp. 1	2	3	4
—	52.0	66.8	86.4	96.2
I*	52.9	68.1	87.7	98.1
α–BHC	50.4	62.5	85.1	99.4
α + I	52.6	70.1	93.7	101.4
β–BHC	48.0	66.3	71.8	—
β + I	46.1	65.2	63.7	—
γ–BHC	26.4	50.3	69.9	85.7
γ + I	23.9	45.3	68.6	80.9
δ–BHC	42.1	66.0	83.4	96.0
δ + I	41.0	65.9	81.6	92.2

* i–Inositol at a level of 0.5 mg/g food.

α, β, and δ isomers were all similar—about 28 mg per rat for the first week, while consumption of the γ isomer was limited to 15 mg. These dosages are in the range of 60 mg/kg/d for the former 3 isomers and 45 mg/kg/d for γ-BHC. The apparent discrepancy between intake and dosage level (28 and 15 mg/rat/wk compared with 60 and 45 mg/kg/d) is explained by the gain in body weight permitted by the α, β, and δ isomers, while the rats consuming γ-BHC lost about 8% of their body weight over the first 4 days of the experiment (from 45.7 g to 42.4 g body weight on the fourth day). In the presence of hyperactivity, a decreased food intake is unexpected. Thus the γ isomer may depress appetite due to a disagreeable odor or flavor, and increase the metabolic needs of the organism without compensatory increase in food intake.

These results clearly indicate that under the conditions of this experiment, this strain of rat does not require dietary inositol; that α- and δ-BHC are relatively inert toxicologically; that the β- and γ isomers are toxic; and that these toxicities are not alleviated by dietary inositol.

The mechanism by which the β and γ isomers exert their toxic effects is poorly understood. The simple alkyl halides such as chloroform and carbon tetrachloride produce well-defined postmortem findings completely absent in the animals receiving these cycloalkyl chlorides. Dallemagne(10) has presented suggestive data which illustrate relative toxicity of the 4 isomers administered by mouth to the rat; an inverse relationship between melting point and toxicity is described. Using Taylor's data(9) he notes the toxicity was greatest with the lowest melting isomer, γ-BHC, and decreased to no toxicity with the highest melting isomer, β-BHC, over a test period of one week. However, our results, obtained over longer periods of time, indicate that β-BHC is more toxic than γ-BHC, a finding which corroborates the work of Fitzhugh *et al.*(11).

Chaix(2) attributes difference in toxicity to variation in solubility of the isomers in different cellular media. The early onset of γ toxicity in our experiment, compared to the longer induction period of the β toxicity, might be due to recognized differences in solubility(9). The lower solubility of the β isomer might retard its transfer across the intestinal membrane, thereby delaying the appearance of toxic signs. Once the critical level for narcosis is reached, further oral intake ceases; however, absorption probably continues, since the pronounced odor of BHC observed at postmortem in the abdominal cavity possibly indicates a residuum of the β isomer yet to be absorbed. Further light has been shed on the mechanism of action by Dallemagne *et al.*(12) who demonstrated in acute toxicity experiments in dogs that there was a synergistic effect in the production of convulsions between the γ isomer and eserine. They interpreted their results as indicating that the γ isomer exerts its convulsive effects by inhibition of cholinesterase. Interestingly enough, counteraction among the isomers has been noted; McNamara and Krop(4) demonstrated that preparation of the animal by the intravenous administration of either the β or δ isomer negated the convulsive effects of the γ isomer on later administration by the same route. Thus, when the role of absorption is eliminated, the convulsive and narcotic actions of these drugs counteract each other, and differences in solubility are probably no longer so important a factor. In our opinion, the opposite effects of the γ and β isomers, convulsive and narcotic, respectively, are best explained not by physical properties but by stereochemical differences between the molecules. The possible isomers of BHC as listed by Bijvoet(7) and Kauer(13) contain a structure analogous to i-inositol, which has been reported to have one polar(14) and 5 equatorial hydroxyl substituents (p,e,e,e,e,e) (7,15). Of the 6 isomers of BHC already

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isolated, the following may be eliminated as analogues if their proposed structures are confirmed: α, since it is apparently a racemate, Cristol(16) having obtained the dextrorotatory enantiomorph; β (e,e,e,e,e,e)(17, 18); γ (p,p,p,e,e,e)(7,8); and zeta (p,e,e, p,e,e)(17). Our data also rule out the first 3 isomers mentioned and the δ isomer on biological evidence. The question of which isomer of BHC is analogous in structure to i-inositol must await further isolation and characterization studies.

The marked differences in physical properties (solubility, melting points, dipole moments) and in chemical reactivities between the hexahydroxy- and hexachlorocyclohexanes are not in accord with the accepted antimetabolite concept of biological competition. Therefore, true specific competitive antagonism will probably not be demonstrated between such physically dissimilar classes of molecules. However, the antithetical physiological properties of the BHC stereoisomers are unique and may therefore become of great interest in elucidating the mechanisms of narcosis and idiopathic convulsive disorders, such as epilepsy.

Summary. The toxicities of the α, β, γ, and δ isomers of hexachlorocyclohexane were not affected by inositol in the rat. The convulsive and narcotic effects of the γ and β isomers are discussed in detail.

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Some Effects of Testosterone Propionate on Mice Irradiated with X-rays.* (17994)

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We have recently demonstrated that administration of desoxycorticosterone acetate after exposure of mice to lethal doses of X-rays reduces the mortality produced by irradition(1,2). It has also been demonstrated that the administration of this hormone protects the liver of mice against radiation induced fatty changes(3) and prevents the radiation induced depletion of sudanophile substances(4) of the adrenal cortex. In order to obtain more complete information on the significance of these findings for the mechanism of total body irradiation, our studies on the pharmacological analysis of this process have been extended to include testosterone propionate. The use of this substance suggested itself because testosterone is not only a chemical relative of desoxycorticosterone but also exercises some similar metabolic effects.

Material and methods. Swiss male white mice (Rockland Farms Stock) 22 g $\pm$ 15% of body weight were used in these experiments. Irradiation took place in the previously described manner(5). The X-ray dose was in all instances 500 r/air (HVL 0.75 mm Cu).

* Based on experiments carried out at the Long Island College of Medicine, N. Y., aided by grants from the John and Mary R. Markle Foundation, New York, and the Schering Corporation, Bloomfield, N. J.

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This dose was administered in one exposure and represents the $LD_{50}/30$ days for these mice under our conditions of exposure. For the hormone treatment a solution of 25 mg of testosterone propionate in 10 cc of sesame oil (Oreton†) was used. Hormone treatment of irradiated animals started on the day of exposure and was given in 6 weekly doses over a period of 2 weeks.

All animals were observed for at least 28 days and some up to 90 days after the start of the experiment. The following experimental groups were used:

A. 34 mice received injections of .25 mg of testosterone propionate and irradiation.

B. 30 mice received injections of .5 mg of testosterone propionate and irradiation.

C. 20 mice received .5 mg testosterone only.

D. 18 mice served as unirradiated untreated controls.

For the study of the influence of testosterone propionate on the X-ray induced mortality rate, their death rate was noted day by day and the mortality curve thus obtained compared with that of 102 mice having been exposed to 500 r/air only.

Autopsy material obtained from the X-rayed and testosterone treated animals, which died during the course of the experiment, served for our study of the influence of testosterone administration on the X-ray induced histological changes. This material was supplemented by organs from 17 mice killed at various time intervals. The organs were fixed in Zenker's solution, embedded in paraffin, sectioned at 4-6 μ and stained with hematoxylin-eosin. In addition, liver and adrenal tissues were also fixed in 10% formalin, sections were made with the freezing microtome and stained with sudan III. The grading system previously described(2,4) was used for the quantitative analysis of the histological changes.

Results. General appearance of the animals: The irradiated animals treated with testosterone developed a ruffled fur about the second or third day after the start of the experiment. This change in the coat of the animals was independent of the dose of testosterone used. At the end of the first week the animals treated with testosterone after irradiation looked very sick in comparison with the animals which had received irradiation only. This change in general appearance was supported by the weight curves. Normal unirradiated animals of our stock gained about 7% of their initial weight during the first week while in 56 animals after exposure to 500 r/air a loss of about 7% of the starting weight was found at that time. The irradiated mice treated with testosterone lost about 11% of their initial weight at the end of the first week. Their general appearance became increasingly worse during the second week. One animal (No. 206/3) was almost bald, when it died on the twelfth day after the start of the experiment and its weight had dropped to 16 g. The general appearance of this group contrasted sharply not only with that of the irradiated control group but also with that of unirradiated animals which received testosterone. The latter showed a slight ruffling of the fur about the fifth day and appeared healthy at the end of the second week.

The influence of testosterone treatment on X-ray induced mortality rate is shown in Fig. 1. In agreement with their general appearance, the testosterone-treated animals showed increased susceptibility to the lethal effects of X-ray. But it appears noteworthy that there was a difference in the onset of death between the 0.25 mg and 0.5 mg dose, even though this difference is not statistically significant. No deaths occurred in the group treated with hormone alone. The study of *the influence of testosterone treatment on the X-ray induced organ changes* indicated no changes in all investigated organs with the exception of the liver, where changes in appearance of sudanophile substances were observed. The data are summarized in Fig. 2. The comparison of the fat accumulation in untreated but irradiated animals (section A) with that found in the group of mice treated with 0.25 mg testosterone and irradiated (section B) indicated no protective action of the hormone in this report. But a similar comparison with the results obtained in the

† Oreton was supplied by the Schering Co. through the courtesy of Dr. E. Henderson.

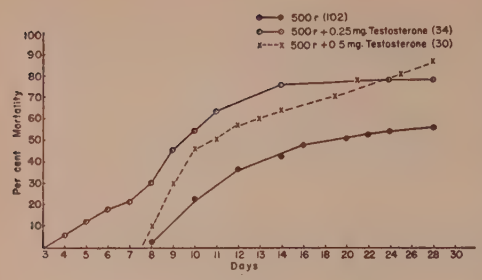

FIG. 1.

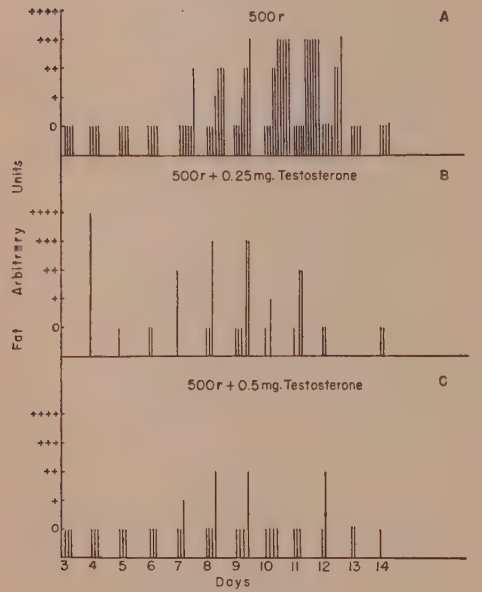

FIG. 2.

group treated with injections of 0.5 mg (section C) shows a definite and statistically significant protective action of the larger hormone dose (probability $p = 0.02$).

Discussion. The foregoing data clearly indicate that administration of testosterone propionate, in contrast to the closely related desoxycorticosterone acetate, enhances the lethal effect of X-rays. Our findings are in agreement with observations made by Edelmann(6) in rats. It appears likely that the antagonistic effect of testosterone and desoxycorticosterone demonstrated by our studies is based on their opposing effects on potassium metabolism. "Potassium diuresis induced by desoxycorticosterone acetate and very important in the repair of adrenal insufficiency is quite opposed to potassium retention induced by testosterone and unlike the negative effects of estradiol usually obtained" (Koch)(7). The similarity in metabolic effects of testosterone and desoxycorticosterone is seen in their effects on fat metabolism. Both steroids are capable of suppressing the radiation-induced fatty changes in the liver. This property was pronounced with the larger testosterone dose used in these experiments, and might possibly explain the somewhat delayed onset of the lethal effect observed in our group B animals. The absence of any protection of the adrenal cortex against radiation-induced depletion of sudanophile substance by testosterone treatment should be emphasized. The impression is given that testosterone administration rather enhances this process. As an indirect support for this contention, some observations made in our group C mice should be mentioned: Our results concerning the testosterone tolerance of unirradiated mice are in agreement with those of Kochakian(8). But histologic examination of the adrenals of 11 mice killed between 4 and 14 days after testosterone administration revealed in 3 animals complete depletion and in 4 a definite reduction of sudanophile substance of the adrenal cortex. In the light of these findings it appears possible to conclude that testosterone, when given in conjunction with irradiation, may augment stimulation of the adrenal produced by irradiation. The worsening in the general appearance of the testosterone-treated irradiated animals, over that of those only irradiated or only hormone-treated could readily be understood on this basis. Assumption of hormone-induced adrenal stimulation permits also under-

6. Edelmann, A. (Brookhaven National Laboratory) private communication. It appears noteworthy that the rats used by Edelmann were about 10 times as heavy as the mice used by us. It is therefore interesting that Edelmann used 5 mg of testosterone in his rats which is exactly 10 times the dose used in our experiments with mice.

7. Koch, F. C., Sex hormones, Jaques Catell Press, Lancaster, Pa., 1942.

8. Kochakian, C. D., *Endocrinology*, 1940, v26, 54.

standing of the opposite effects of estradiol administration prior to or after irradiation (9,10). Finally, the observations concerning the action of testosterone administered to animals exposed to lethal doses of X-ray seem to support the opinion of Koch(7) that "neither testosterone nor the estrogens serve to repair adrenal failure."

Our findings concerning increased radiosensitivity in total body irradiation after testosterone administration appear interesting also from the point of view of general radiation biology, inasmuch as they offer an interesting parallelism to observations concerning thyroxin:

In previous studies we have been able to demonstrate that thyroxin increases radiosensitivity of the skin to X-ray(11) as well as to ultraviolet rays(12,13), and Blount and Smith recently demonstrated an increased radiosensitivity in total body X-irradiation (14). We have also been able to demonstrate that skin radiosensitivity increases significantly at puberty in both sexes(12) when sex hormones are produced more abundantly. From the previous and the present studies it appears that hormones in general may alter radiosensitivity locally and also as far as the response of the entire body is concerned, and that this change in general radiosensitivity is not specific for the type of radiation used over a large part of the spectrum(15). This appears to be of interest not only from a theoretical point of view but also from the standpoint of clinical medicine, because of the now generally accepted use of sex hormones in the treatment of certain malignant tumors.

Summary. 1. Administration of testosterone propionate in daily doses of 0.25 and 0.5 mg, respectively, following exposure of mice to the LD_{50} of X-ray, up to 14 days, markedly increased the lethal effect of irradiation. 2. No changes in the X-ray induced organ effects were accomplished in all investigated organs with the exception of the liver. Radiation induced accumulation of sudanophile fat in this organ was definitely suppressed with larger dose of testosterone. 3. Radiobiological and some clinical implications of these findings are discussed.

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Formation of the β-Carbon of Serine from Formaldehyde.*† (17995)

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Sakami(1) has demonstrated that formate carbon can serve as a source of the β-carbon of serine. Since the conversion of glycine to serine had also been demonstrated(1-3) it was suggested(1) that formate or a formate derivative condensed with glycine to form serine. This is a reversal of the mechanism suggested by Shemin(4) for the conversion of serine to glycine. Kruhoffer(5) further investigated this problem and obtained evidence indicating that formate itself was not a direct precursor of the β-carbon of serine. He found that in a rat liver homogenate carboxyl-labeled glycine was utilized for serine synthesis, whereas the labeled formate was not. This absence of labeling of the β-carbon of serine when C^{14}-formate was used was not due to dilution of the isotopic formate since formate isolated at the end of the experiment had almost as high a specific activity as the added compound. Apparently the enzyme system involved in the conversion of formate to the direct precursor of the serine β-carbon was absent in this preparation, this carbon being supplied by endogenous reactions.

The possibility has been investigated that formaldehyde condenses directly with the α-carbon of glycine to form serine. This has been studied by determining the ability of rat liver homogenate (prepared in the manner of Kruhoffer) to incorporate the C^{14} of labeled formaldehyde into the β-carbon of the serine.

Methods. Labeled formaldehyde was synthesized by reduction of $C^{14}O_2$ with lithium aluminum hydride(6) and oxidation of the resulting methanol to formaldehyde by admixture with dry air and passage over a vanadium molybdenum oxide catalyst(7). The hot vapors were then passed over calcium chloride to remove water formed during the oxidation of the methanol. This prevented moist formaldehyde from polymerizing on the walls of the tubing. The calcium chloride also served to remove unoxidized methanol. The formaldehyde was trapped in water; it contained a trace of methanol (less than 2%) but no formate, and possessed a specific activity of 2.5×10^6 counts per minute per mg carbon. Experiments were conducted in which C^{14}-formate, C^{14}-formaldehyde, and C^{14} carboxyl-glycine were incubated separately with a rat liver homogenate prepared from one part (wet weight) of rat liver and 2.5 parts of modified Krebs-Henseleit medium(8). The homogenate was freed of unbroken cells by low speed centrifugation. Nine ml of homogenate were added to each Warburg vessel together with other substances (Table I) in 0.3 ml volume, making the total volume 9.3 ml. Incubation was carried out at 38°C for 90 minutes in 95% oxygen and 5% carbon dioxide.

After the incubation, 500 mg DL-serine were added as a carrier to each vessel. The homogenates were then deproteinated with trichloracetic acid and the trichloracetic acid

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TABLE I.
Protocol of First Group of Experiments.

Exp. No.	Labeled compound	μM	Total activity*	Non-labeled compounds† Glycine, μM	Isolated serine COOH-carbon‡	α-carbon‡	β-carbon‡
1	Na formate	30	1.43×10^6	30	.00	.00	.06
2	Formaldehyde	35	1.07×10^6	30	.02	.01	.62
3	Formaldehyde + heat inactive homogenate	35	1.07×10^6	60	.00	.00	.00
4	COOH-labeled glycine	13	7.7×10^5	—	17.7	.01	.01

* Counts per min. counted as $BaCO_3$.
† 3.0 mg DL-serine were added to each vessel during the incubation.
‡ % of total C^{14} activity added.

TABLE II.
Protocol of Second Group of Experiments.

Exp. No.	Labeled compound used	μM	Total activity*	Non-labeled compounds added† Na formate μM	Formaldehyde μM	Methanol μM	% of C^{14}‡ recovered in serine
1	Na formate	30	1.43×10^6	—	34	34	.07
2	Formaldehyde	35	1.07×10^6	34	—	34	.59
3	Methanol	33	7.2×10^5	34	34	—	.11

* Counts per min. counted as $BaCO_3$.
† 3.0 mg DL-serine and 33 μM glycine added to each vessel during the incubation.
‡ Counted directly on serine (corrected to values for $BaCO_3$).

was removed by ether extraction. The volume of the solution was adjusted to 30 ml and 20 ml of ethanol were added. The samples were heated on a water bath, allowed to cool slowly, and filtered to remove the glycogen. The filtrates were then evaporated to 5 ml and 12.5 ml of ethanol were added to precipitate the serine. This was recrystallized to constant specific activity from ethanol-water solution (6 recrystallizations). The serine was then converted into the p-hydroxyazobenzene sulfonate. The activity of this derivative remained unchanged on recrystallization. In the experiment in which carboxyl-labeled glycine was present, to reduce the possibility of contamination of the isolated serine by labeled glycine, 0.5 mM of non-isotopic glycine was added to the serine after the sixth recrystallization and subsequently removed as the 5-nitronaphthalene-1-sulfonate. Excess 5-nitronaphthalene-1-sulfonic acid was removed from the serine solution by adsorption on the ion exchange resin, Amberlite IR-4B, and the serine was then made into its derivative as described above. The serine isolated and purified in this manner was degraded according to the method of Sakami(1) whereby the carboxyl, alpha, and beta carbons are separately converted to carbon dioxide. The CO_2 samples were counted as barium carbonate and the overall accuracy of the C^{14} determinations was approximately 5%. In the experiment in which labeled formate was used, the C^{14} of the formate remaining at the conclusion of the experiment was determined by the following procedure. Carrier formate was added before the serine isolation. It was then removed by ether extraction and converted to CO_2 by mercuric chloride oxidation. The radioactivity of the carbon dioxide was determined as described above.

Experiments were also done to compare formaldehyde and formate uptake into serine with that of C^{14}-methanol. This was done principally to rule out the possibility that the labeling of serine from C^{14}-formaldehyde was due to the small amount of contaminating

C^{14}-methanol. The experiments (Table II) were conducted in a similar manner to the previous ones. The carrier serine was recrystallized to constant activity and counted directly for C^{14} activity to measure the relative uptake of isotope from each of the 3 different labeled one-carbon compounds while in the presence of the unlabeled other 2.

Results. The results of the first group of experiments are shown in Table I. The C^{14} of the formaldehyde labeled the β-carbon of the non-protein serine. In confirmation of Kruhoffer's results there was very slight incorporation of isotope when labeled formate was added although 12% of the added C^{14}-formate was present at the conclusion of the experiment. The C^{14} from the carboxyl-carbon of labeled glycine was more extensively incorporated into the serine than was the C^{14} from the formaldehyde.

In the second group of experiments (Table II) testing C^{14}-labeled formate, formaldehyde, and methanol each individually but in the presence of approximately equal amounts of the other one-carbon compounds which were unlabeled, it was found that C^{14}-formaldehyde labeled serine eight times more than did C^{14}-formate and five times more than did C^{14}-methanol. This would indicate that in the first group of experiments the small amount of contaminating C^{14}-methanol in the C^{14}-formaldehyde solution was not responsible for most of the C^{14} entering into the serine.

Discussion. In our experiments with liver homogenate more C^{14} was incorporated into the non-protein serine β-carbon from formaldehyde than from formate or methanol. Apparently formaldehyde is utilized more readily for the synthesis of serine than is formate or methanol. The isotope from C^{14} carboxyl-glycine, however, was incorporated into serine to a greater extent than that from the formaldehyde; this difference in isotope incorporation between formaldehyde and glycine lends itself to two possible explanations. Formaldehyde might condense directly with glycine in the biosynthesis of serine but there may be a greater dilution of the C^{14}-formaldehyde from unlabeled formaldehyde sources as compared to the glycine dilution. Formaldehyde in mammalian liver has been shown to arise from dimethylglycine(9) and sarcosine(9,10), both of which have been implicated in choline metabolism(11,12), and from the α-carbon of glycine, the methyl carbons of methionine and choline, and the β-carbon of serine(13). These compounds could be the source of dilution of added C^{14}-formaldehyde.

Another possible explanation for the smaller amount of isotope uptake from labeled formaldehyde as compared to that from labeled glycine might be that the C^{14}-formaldehyde, itself, is not diluted to any significant extent but that an intermediate to which formaldehyde must be converted prior to the condensation with glycine is diluted by endogenous sources of this intermediate. Possible sources of this intermediate could be compounds that have been shown to be precursors of the β-carbon of serine. The α-carbon of glycine(14,15), methyl groups of choline(16,13) and methionine(13), and the methyl groups of acetone(17) have been demonstrated in isotope tracer experiments to be sources of the β-carbon of serine. Since these serine β-carbon precursor compounds, with the exception of acetone, are either the same or metabolically related to the same compounds that are known formaldehyde sources it is not unlikely that a common intermediate exists derived from these compounds which is in equilibrium with the β-carbon of serine and with formaldehyde. At the present time there is no data which permits a choice between either of these explanations for the lesser incorporation of formaldehyde isotope into serine as compared to that from the carboxyl-carbon of glycine. Nevertheless, it

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is probable that formaldehyde is closer to the immediate precursor of the β-carbon of serine than either formate or methanol.

Summary. C^{14} from labeled formaldehyde was incorporated into the β-carbon of serine greater than that from labeled formate or methanol. The indications are that formaldehyde is the more direct precursor of the β-carbon of serine.

Addendum. As an extension of the work by Welch and Sakami(18) in which they found that in the intact rat and in liver slices there was incorporation of C^{14} from formate into the methyl groups of methionine and choline we have made some preliminary studies with C^{14}-formaldehyde. Employing the same rat liver homogenate used in these serine studies we have found evidence that formaldehyde is converted into the methyl group of methionine much more extensively than is formate.

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Preparation of Water-Soluble Combination Products of Gossypol and their Toxicity to Aquarium Fish.* (17996)

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The purpose of the investigations here described has been to make combination products of gossypol(1-3) with naturally occurring materials which would have physical properties different from those of the original material and to determine the effect of such changes on some physiological action of gossypol. Gossypol was tested on aquarium goldfish and sharp differences in physiological activity between the original gossypol and the combination products were obtained.

Materials and methods. Pigment glands were prepared from cottonseed by the gland fractionation process(4,5). Gossypol was prepared by acetone extraction of pigment glands and crystallization from a mixture of diethyl ether and light petroleum naphtha (6). Peanut protein was prepared by alkaline extraction of oil-free peanut meal followed by acid precipitation of the protein (7). The casein, amino acids, dextrose, and starch used in the experiments were commercial products.

The general procedure for preparation of the combination products involved two steps, namely: (1) Gossypol and the material to which it was to be combined were solubilized in aqueous solution of pH of approximately 11 in the shortest possible time. (2) The solubilized material was neutralized with hydrochloric acid and dried in the frozen state (lyophilized).

Ten grams of purified peanut protein was added to one liter of distilled water and the

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pH was adjusted to 8.5 by use of dilute aqueous sodium hydroxide. While the solution was being stirred 10 g of gossypol and more sodium hydroxide were added until a pH of 11 was obtained. Immediately thereafter hydrochloric acid was added until the pH was reduced to approximately 7 after which the mixture was frozen and lyophilized. A yield of 20 g of light yellow colored fluffy product which was soluble in water or buffer solution of pH 7.0 to the extent of 10% was obtained. Several other preparations were made in which the ratio of gossypol to peanut protein was varied. When large quantities of this preparation are required it is of advantage to precipitate the gossypol-protein product with hydrochloric acid by lowering the pH to 4.5. This product is removed by centrifugation and can then be transferred with a small amount of water to the lyophilizing apparatus and dried. In order however to effect solution of the dry product in water it is necessary to adjust the pH to 7 with alkali. Water-soluble gossypol-glycine and gossypol-lysine products were prepared by combining equal weights of gossypol and the amino acids according to the above mentioned procedure. Similar procedures were used in preparing combination products with dextrose and starch.

Physiological experiments. Two to 8 goldfish were placed in the test and control solutions contained in wide-mouthed glass jars 6½" high and 6" in diameter. Each 2 fish were in approximately one liter of solution. Air was bubbled through each solution at the same rate, the pH was adjusted to 7.0 in each case, and the temperature was maintained at 75° F. The time of exposure of the fish to the test solutions until death ensued has been recorded as the average value of the time taken for the death of all experimental animals in a test solution. The range of variation of death time in any given solution did not exceed 20%.

Testing of crystalline gossypol. A mixture of gossypol in water at a concentration of 0.04% gossypol and adjusted to pH of 7.0 was violently agitated. Nevertheless the gossypol remained upon the surface of the water after agitation had ceased. Goldfish placed in this test solution lived for seven days and upon transfer to fresh water continued to be healthy. Combination-product formation was suggested by the following experiment. Gossypol was added to a .005% solution of peanut protein and the mixture was stirred. The pH of the solution was increased to 10.3 by use of dilute sodium hydroxide solution and then reduced to neutrality by use of hydrochloric acid. Under such conditions of preparation, the gossypol remained in solution throughout the test and in a concentration range of 1 to 5 parts per 100,000 parts of 0.005% protein solution was quite lethal to fish within 24 hours. Control solutions containing an equivalent amount of protein and sodium chloride were not harmful to the test animals. Physical suspension of the gossypol does not put it in a condition wherein it is toxic to fish. It is necessary to completely solubilize the gossypol and maintain it in solution with protein in order to obtain the toxic effect.

Water-soluble combination products of gossypol. In Table I are shown the extractable gossypol contents of each of the freshly prepared samples of combination products and their toxicities to aquarium fish. An aqueous solution of gossypol-peanut protein that produced death of fish within 1 hour at a concentration of 1 part of the material per 20,000 parts of water was stored for 48 hours at 38° F. After this period the solution still had its original toxicity and gossypol content. Control solutions containing an equivalent amount of protein, amino acid, starch, dextrose, and sodium chloride were not harmful to the test animals.

At a concentration of one part of pigment glands to 2000 parts of water, the glands were lethal to the fish. However, such solutions had only about one-half of the toxicity of solutions of combination product of gossypol which were of the concentration of one part of product to 20,000 parts of water. Results of these investigations are shown in Table II.

It is quite clear from a comparison of the results in Tables I and II that the combina-

TABLE I.
Toxicities to Goldfish of Water-soluble Combination Products of Gossypol with Materials of Natural Origin.

Grams of combination product per ml aquarium bath	Combination product: Gossypol-peanut protein A	Gossypol-peanut protein B†	Gossypol-peanut protein C‡	Gossypol-casein	Gossypol-starch	Gossypol-dextrose	Gossypol-glycine	Gossypol-lysine
	Extractable gossypol content of dry product,* %							
	46.1	40.0	52.2	41.0	36.9	39.2	28.4	44.8
	Exposure of fish to test sol. before death —(hr)							
5×10^{-5}	1.	1.7	1.3	2.	1.4	1.7	1.7	2.
2.5×10^{-5}	1.7	4.4	1.6	<24.	2.3	2.4	3.4	<24.
1.6×10^{-5}	3.6	6.1	3.1	<24.	—	2.9	2.7	<24.
1.25×10^{-5}	4.0	<24.	<24.	<24.	—	—	<24.	<24.
1×10^{-5}	<24.	<24.	<24.	<24.	—	—	<24.	>24.

* Calculated on dry-weight basis. Percent extractable gossypol determined by application of the antimony trichloride spectrophotometric method applied to chloroform solutions prepared from aqueous ethanol extracts(8).

† This sample was lyophilized at pH 4.5; all other samples in Table I were lyophilized in aqueous solution of pH 7.0.

‡ Two parts of gossypol were combined with one part of protein; in all other samples in Table I, gossypol was combined with an equivalent weight of material of natural origin.

TABLE II.
Toxicities of Separated Cottonseed Pigment Glands to Goldfish.

Lot of pigment glands	Extractable pigment content of glands*: Gossypol,† %	Gossypurpurin,‡ %	G of pigment glands per ml of aquarium bath sol.	Exposure of fish to test sol. before death (hr)
A	39.6	0.83	5×10^{-4}	3.9
			2.5×10^{-4}	5.3
			1.7×10^{-4}	<24.0
B	33.8	1.74	5×10^{-4}	6.3
			2.5×10^{-4}	<24.0
C	29.9	1.16	5×10^{-4}	5.7
			2.5×10^{-4}	<24.0

* Calculated on basis of weight of pigment glands.

† Determined by application of the antimony trichloride spectrophotometric method to chloroform solutions prepared from aqueous ethanol extracts of the glands(8).

‡ Calculated on basis of $E^{1\%}_{1\ cm}$ at 568 m_μ of chloroform extracts of glands(3).

tion products of gossypol are much more toxic to goldfish than the pigment glands. Although the gossypol in pigment glands is not as active toward goldfish as that in the combination products it is much more active than pure gossypol itself. This observation would indicate that gossypol, as it exists in the pigment glands, is in combination with some of the other material in the glands rather than completely in the free state. Such combination is of a nature that renders the gossypol either completely soluble or at least dispersible in a very finely divided state in aqueous medium.

Discussion. The exact nature of the reaction of gossypol with the materials used in making the water-soluble products is not yet known. The fact that gossypol contains hydroxy groups and carbonyl groups would indicate that this material is capable of combining with the amino groups of proteins and amino acids, and the hydroxy groups of carbohydrates. It is known that when cottonseed meal is cooked and the gossypol re-

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leased from the pigment glands, some of it reacts with the reactive groups in the meal and is "bound." Under such circumstances the gossypol is detoxified. For the combination products described in this publication the gossypol is rendered more toxic by a process which maintains it in solution. A more thorough study of the reactions of gossypol with amino acids, proteins, and carbohydrates, and of the effect of storage and heat on such products would undoubtedly clarify the nature of this combination product and probably throw light on the reaction that takes place when cottonseed or cottonseed meats are cooked in order to detoxify the gossypol.

Summary. (1) Gossypol was combined with proteins, amino acids, and carbohydrate materials by a procedure involving mixing of the gossypol and other combining substances in alkaline solutions and subsequent lyophilization of the neutralized frozen solutions. (2) Crystalline gossypol when added to the aquarium bath had no visible effect upon goldfish. The water-soluble combination products of gossypol were toxic to fish in concentrations as small as one part of gossypol product to a hundred thousand parts of water. (3) Separated pigment glands were toxic to fish but were significantly less toxic than the water-soluble combination products of gossypol.

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Lipotropic Effects of Liver Extract, Vitamin B_{12} and Choline.* (17997)

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It has been reported that crude liver extract exerts a lipotropic effect and will prevent fibrosis of the liver when administered to rats receiving a high fat diet(1). The lipotropic effect of the liver extract did not seem to be due to choline content. In fact, other supplements which did not give protection against dietary induced liver injury had a higher content of choline than was present in liver extract(2). In further investigations a vitamin B_{12} concentrate, prepared from liver, also exerted a definite effect in preventing accumulation of fat in the liver of rats(3). In the present study the relationship between dosage of liver extract and lipotropic effect was studied. Observations were also made on the possible lipotropic effect of combinations of choline, inositol and folic acid, which are present in small amounts in liver extract and vitamin B_{12} concentrate. Crystalline vitamin B_{12} alone and in combination with small amounts of choline was also observed for lipotropic activity.

Methods. Male Sprague-Dawley rats weighing between 120 and 150 g were used. They were fed the high-fat diet (51% lard) and the control diet (6% lard) recently described(1). These diets were fed for the periods listed in the tables. When the animals were placed on the synthetic diet, injections of the supplements were begun. All supplements were administered subcutaneously except in one experiment with liver extract as noted in Table I. At the end of the study sections were taken from the left lobe of the liver and stained with hematoxylin and eosin or with Sudan III for fat. The remainder of the liver was analyzed for total

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TABLE I.
Effect of Subcutaneous and Oral Liver Extract on Hepatic Changes of Rats Fed a High-Fat Diet.

No. of rats	Diet	Liver extr., cc 3x week, γ	Avg wt gain, g	Avg liver wt, g/100 g rat	No. animals with hepatic fatty change 0	1+-2+	3+-4+	Avg liver fat, %
			Exp. 1—66 days					
4	Normal	—	161± 3.1	4.0±0.37	3	1	0	5.6±1.08
8	H.F.*	—	43± 9.7	8.5±0.62	0	0	8	24.8±0.59
4	”	1.0 (s.q.)	77± 4.7	4.4±0.46	2	1	1	8.4±1.27
			Exp. 2—150 days					
6	Normal	—	172± 9.3	3.9±0.12	3	3	0	7.5±0.35
8	H.F.	—	63± 7.7	6.8±0.55	0	1	7	14.6±1.52
6	”	1.0 (s.q.)	114±10.9	4.9±0.25	0	5	1	9.7±1.21
			Exp. 3—92 days					
12	Normal	—	135± 4.9	3.9±0.08	9	3	0	6.8±0.49
9	H.F.	1.0 (oral)	51± 7.7	4.8±0.22	1	7	1	11.4±1.32
10	”	.75 (s.q.)	71± 6.1	4.1±0.12	3	7	0	14.8±1.54
10	”	.5 ”	51± 6.5	5.5±0.41	1	8	1	16.3±1.30
10	”	.25 ”	72± 5.0	6.9±0.44	0	3	7	20.3±1.74
12	”	—	9± 6.3	8.7±0.58	0	3	9	18.7±1.39

* H.F. = High fat diet.

Grading of fatty change:
0—None or only a rare large droplet.
1+—A few large droplets in each lobule.
2+—More than 1+, but half of lobule involved.
3+—Over half of lobule involved, but some cells fat free.
4+—Almost no fat free cells.

S.E. of the mean = $\sqrt{\frac{\Sigma d^2}{n(n-1)}}$.

TABLE II.
Effect of Crystalline Vitamin B_{12} on Hepatic Changes of Rats Fed a High-fat Diet.

No. of rats	Diet	Cryst. B_{12} 3x week, γ	Avg wt change, g	Avg liver wt, g/100 g rat	No. animals with hepatic fatty change 0	1+-2+	3+-4+	Avg liver fat, %
			Exp. 1—29 days					
6	H.F.	—	+ 55± 6.1	8.1±0.47	0	0	6	31.5±1.18
4	”	0.2	+ 42± 3.3	5.8±0.52	1	1	2	21.4±2.97
			Exp. 2—30 days					
10	Normal	—	+ 77± 5.7	4.1±0.13	9	1	0	4.8±1.20
10	H.F.	—	+ 26± 5.2	4.7±0.20	0	8	2	17.5±1.49
10	”	1.0	+ 33± 4.1	5.6±0.36	1	2	7	14.1±1.77
			Exp. 3—64 days					
7	Normal	—	+151±10.2	3.6±0.25	5	2	0	8.4±1.05
8	H.F.	—	— 6± 5.7	8.1±0.74	0	2	6	26.7±1.83
10	”	1.0	+ 34± 4.2	8.6±0.53	0	1	9	26.2±2.54
10	”	4.0	+ 23± 6.9	8.4±0.49	0	3	7	24.6±0.83
			Exp. 4—30 days					
8	Normal	—	+113± 4.2	4.4±0.12	6	2	0	6.8±0.31
8	H.F.	—	+ 23± 5.5	8.1±0.39	0	0	8	28.8±1.03
10	”	2.5*	+ 19± 3.5	7.8±0.38	0	0	10	27.3±1.12

* Dose administered 6x week.

TABLE III.
Effect of Crystalline Vitamin B_{12} and Choline on Hepatic Changes of Rats Fed a High-Fat Diet for 30 Days.

No. of rats	Diet	Supplement administered 3x wk: Cryst. B_{12} γ	Supplement administered 3x wk: Choline, mg	Avg wt gain, g	Avg liver wt g/100 g rat	No. animals with hepatic fatty change: 0	1+-2+	3+-4+	Avg liver fat, %
10	Normal	—	—	77±5.7	4.1±0.13	9	1	0	4.8±1.20
10	H.F.	—	—	26±5.2	4.7±0.20	0	8	2	17.5±1.49
9	,,	1	.5	40±4.0	5.7±0.39	0	5	4	19.0±1.53
10	,,	—	.5	18±6.2	6.4±0.37	0	4	6	26.6±2.01
10	,,	1	1.	47±5.0	5.6±0.34	0	5	5	23.5±2.26
9	,,	—	1.	24±4.6	5.7±0.39	0	5	4	23.4±1.83
10	,,	1	2.	40±4.2	5.4±0.42	0	7	3	18.9±2.24
10	,,	—	2.	20±4.5	6.9±0.39	0	3	7	26.1±2.38

fat by a modification of the method of Outhouse and Forbes(4).

Results. Crude liver extract. Crude liver extract, containing 1 U.S.P. unit of antianemic principle per cc was administered 3 times a week subcutaneously to rats receiving a high-fat diet for periods of 66 and 150 days (Table I). The liver extract exerted a lipotropic effect and fibrosis, which developed in 6 untreated rats fed the high-fat diet for 150 days, was also prevented by administration of liver extract, confirming results previously reported. The weight of the liver of the treated rats was also in the range of that observed in the normal control animals. Another group of 4 rats, not shown in Table I, received choline, 2 mg per rat 3 times a week subcutaneously, with the high-fat diet for 66 days. This is a greater amount of choline than is supplied by the liver extract. No significant effect of this amount of choline was obtained, the total liver fat averaging 19.0 ± 3.54%.

Dosage of liver extract and lipotropic effect. Animals receiving the high-fat diet received various doses of liver extract subcutaneously 3 times a week (Table I). As the dose was decreased the amount of fat in the liver rose progressively in each group. Similarly, the average liver weight in grams per 100 g of rat weight increased and more animals showed an increase in liver fat histologically. Thus, one cc of crude liver extract administered subcutaneously 3 times a week represents approximately the minimum effective dose of this material under the conditions of these experiments. One group in this study received one cc of liver extract 3 times a week orally. The oral administration of the liver extract showed a definite lipotropic effect, as evidenced by a decreased total liver fat, reduction in liver weight, and prevention of histological fatty changes (Table I). However, the oral administration of the liver extract is slightly less effective than the same dose of this material administered subcutaneously.

Supplements of choline, inositol and folic acid. The liver extract, and also the vitamin B_{12} concentrate previously reported(3), con-

4. Outhouse, E. L., and Forbes, J. C., *J. Lab. and Clin. Med.*, 1939, v25, 1157.

tain small amounts of choline and folic acid. The concentration of inositol in these supplements is unknown but is quite small. To test the possibility that a combination of small amounts of choline, inositol and folic acid might exert a lipotropic effect, these supplements were administered in combination to rats receiving a high-fat diet. Ten rats received the high-fat diet plus the subcutaneous injection 3 times a week of choline 1 mg, inositol 1 mg, and folic acid 2 μg. Another group of 9 rats received these same supplements in half of the above concentrations. The animals were autopsied on the 92nd day of this study and liver fat of these treated animals was high (20.1% $\pm$ 0.58%) and not significantly different from that found in untreated high-fat controls animals (18.7% $\pm$ 1.39%). Similarly these supplements failed to prevent the histological fatty change in the liver or the increase in liver weight that is obtained when a high-fat diet is fed.

Crystalline vit. B_{12}. Since crystalline vitamin B_{12} is present in liver extract and vitamin B_{12} concentrate, the possible lipotropic effect of this substance was studied. In an initial study, 0.2 μg of vitamin B_{12} appeared to have a slight lipotropic activity. However, this effect probably represents a variation among the rats, as on further study with higher doses of vitamin B_{12} lipotropic activity could not be demonstrated. This was true even when 2.5 μg of crystalline vitamin B_{12} was administered subcutaneously 6 times a week (Table II). Crystalline vitamin B_{12} failed to prevent the fatty change as seen histologically, the increase in liver fat determined chemically, and the increase in liver weight.

Combination of crystalline vit. B_{12} and choline. Inasmuch as vitamin B_{12} has been demonstrated to exert a sparing effect on choline as measured by the growth of the chick(5,7) or the prevention of renal hemorrhagic necrosis in rats(6,7), it was important to test such a combination for lipotropic activity. Choline was administered with and without crystalline vitamin B_{12} in the doses in which it is present in liver extract and vitamin B_{12} concentrate. The vitamin B_{12} dose was kept constant at 1 μg 3 times a week. At each dose level the supplements of choline alone or in combination with crystalline vitamin B_{12} did not decrease the amount of liver fat as determined chemically below that obtained in untreated animals receiving the high-fat diet. These combinations of vitamin B_{12} and choline were also without effect in preventing the histological changes in the liver. The animals receiving both choline and crystalline B_{12} did, however, gain on the average 20 g more in weight than the animals receiving the choline alone (Table III).

Discussion. The lipotropic effect of liver extract was confirmed. One cc of crude liver extract subcutaneously 3 times a week represents about the minimum effective lipotropic dose of this material, under the conditions of this study. As the dose is lowered below this level the protective effect progressively decreases. Liver extract is also effective orally and one cc 3 times a week is only slightly less effective than the subcutaneously administered material. Liver extract also prevented fibrosis, confirming earlier reports(1,2). Recently it has also been noted to be of value in the treatment of experimental dietary cirrhosis in rats(8). In the same study the authors noted that crude liver extract administered orally seemed to enhance the effect of casein and methionine supplements.

The combination of choline, inositol and folic acid that was used did not exert any lipotropic effect. The dosage of these materials was in the range of that supplied by the liver extract used in these studies and by the vitamin B_{12} concentrate used in an earlier report. Thus the lipotropic effect of liver extract or vitamin B_{12} concentrate is not due to a combination of the small amounts of choline, inositol and folic acid present in these materials. Crystalline vitamin B_{12} alone was also without lipotropic effect (Table II). György and Rose(9) have noted that crystal-

5. Schaefer, A. E., Salmon, W. D., and Strength, D. R., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 202.

6. Schaefer, A. E., Salmon, W. D., and Strength, D. R., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 193.

7. Schaefer, A. E., Salmon, W. D., Strength, D. R.,

8. György, P., and Goldblatt, H., *J. Exp. Med.*, 1949, v90, 73.

line vitamin B_{12} is without lipotropic activity in rats fed a high-fat diet. They did, however, obtain a partial lipotropic effect of crystalline vitamin B_{12} when administered to rats receiving a low-fat-low-protein diet, although the effect was less than that obtained with methionine. A combination of methionine and vitamin B_{12} did not further prevent the fatty changes in the liver.

A sub-optimal amount of choline, equal to or greater than that present in liver extract and vitamin B_{12} concentrate, was also without lipotropic effect. Crystalline vitamin B_{12} in combination with choline did not exert any sparing effect on the choline as judged by the failure to prevent an increase in liver fat (Table III). Thus, although vitamin B_{12} has a choline sparing effect on the growth of chicks or in the prevention of hemorrhagic renal necrosis in rats(5,6,7), it is without sparing action in preventing hepatic injury in rats receiving a high-fat diet with casein as the source of protein. As the lipotropic activity of liver extract or vitamin B_{12} concentrate is not due to the factors discussed above, the effect of these materials must be due to other known or unknown agents which influence the synthesis or transport of methyl groups. The possible effects of combinations of crystalline vitamin B_{12} and sub-optimal amounts of folic acid, with or without choline, are being studied.

Summary. With the diet employed, one cc of crude liver extract administered subcutaneously 3 times a week represents the approximate minimal effective dose of this material for lipotropic activity. The same dose of liver extract administered orally also has lipotropic activity but slightly less than that obtained on subcutaneous administration. The lipotropic effect of crude liver extract or vitamin B_{12} concentrate is not due to the small amounts of choline, inositol and folic acid present in these materials. Crystalline vitamin B_{12} alone is without lipotropic effect. Small amounts of choline, alone or in combination with crystalline vitamin B_{12}, also failed to prevent fatty changes in the liver.

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9. György, P., and Rose, C. S., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 372.

Effect of B Complex Vitamins on Liver and Heart Glycogen Levels of Hyperthyroid Rats. (17998)

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The importance of some of the members of the B-complex vitamins in partially counteracting the growth-retarding effect of experimental hyperthyroidism in immature rats is generally recognized. When a high B-complex diet is further supplemented with dried liver or a water-insoluble residue of liver, animals grow at a nearly normal rate in spite of the hyperthyroid condition(1-5). Since hyperthyroidism usually leads to a marked reduction in the concentration of glycogen in the heart and liver, it was decided to determine whether these supplements which so favorably influence the growth rate of the immature animal, would also reduce the effect of hyperthyroidism on the liver and heart glycogen concentrations.

Method. Young albino rats averaging 55 g were put into individual cages and fed the various diets shown in Table I. Food intake was unrestricted but a daily record of food

1. Ershoff, B. H., PROC. SOC. EXP. BIOL. AND MED., 1947, v64, 500.

2. Ershoff, B. H., *Arch. Biochem.,* 1947, v15, 365.

3. Betheil, J. J., Wiebelhaus, V. D., and Lardy, H. A., *J. Nutrition,* 1947, v34, 431.

4. Ershoff, B. H., PROC. SOC. EXP. BIOL., AND MED., 1949, v71, 209.

5. Ershoff, E. H., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 459.

TABLE I.
Composition of the Various Diets.

	Diet 1	2	3	4
	g	g	g	g
Casein, vit. free	200	200	220	300
Sucrose	703	703	683	603
Crisco	50	50	50	50
Cellu flour	20	20	20	20
Salt mixture Wesson(6)	27	27	27	27
Choline chloride	1	1	1	1
Inositol	1	1	1	1
	mg	mg	mg	mg
Thiamine HCl	1	10	10	10
Riboflavin	2	20	20	20
Niacin	3.7	37	37	37
Ca pantothenate	3.7	37	37	37
Pyridoxine HCl	1.5	15	15	15
PABA	150	150	150	150
Biotin	—	—	2	2
Folic acid	—	—	2	2

Diet 5 was similar to Diet 4 except that it contained 100 g of liver meal instead of an equal quantity of casein.

One drop of reinforced oleum percomorphum (Mead Johnson) was given to each rat 5 days a week and one drop of wheat-germ oil twice a week. Each gram of the oleum percomorphum preparation contained 60,000 U.S.P. units of vit. A and 8,500 U.S.P. units of vit. D.

consumption was kept. At the end of the experimental period, which varied from 17 to 41 days, the animals, unstarved, were beheaded and allowed to bleed for about 15 seconds. The heart, after making incisions in it and squeezing out the blood, was put rapidly into a weighed test tube containing 30% potassium hydroxide. Two sections from the left lobe of the liver were treated similarly. Glycogen was then determined by the method of Goode *et al.*(7) with minor modifications. In using this method, we have found it advantageous to purify the glycogen by solution in water and reprecipitation with alcohol prior to acid hydrolysis. Studies on the rate of glycogenolysis in the livers of decapitated rats have shown that it is relatively slow. Heart glycogen disappears more rapidly, and for this reason the heart was removed first in order to get it into the potassium hydroxide as quickly as possible. Duplicate slices from the left lobe of the liver were analyzed in all cases. With few exceptions, the glycogen content of the 2 sections showed good agreement. The values given in Table II are the averages of the 2 determinations.

Results. It will be seen that the concentration of glycogen in both the heart and the liver of the hyperthyroid rat was low in practically all cases. Although the administration of diets high in the crystalline B-complex vitamins, especially when supplemented with defatted liver or the water-insoluble residue of liver, exerted a very favorable effect on the growth rate of the hyperthyroid animal, they did not influence demonstrably the effect of the hyperthyroid condition on the liver and heart glycogen concentrations. Some of the decrease in the concentration of glycogen in the heart may be secondary to the marked cardiac hypertrophy. The concentration of glycogen in the heart of the non-thyroid-fed control rats, in all experiments except No. 14, tended to be considerably lower than we usually find in our stock animals on Rockland rat pellets. No explanation for this difference is apparent at the present time. The addition of 30 μg of vitamin B_{12} per kilo of high-vitamin B-complex diet did not exert a demonstrable effect on the growth rate of the hyperthyroid animals. This finding is consistent with those reported previously by Ershoff(4,5). In other experiments we have found little stimulatory effect on growth from the administration of a 1:20 liver concentrate known to be active in the treatment of pernicious anemia. This also did not exert a favorable influence on the glycogen concentration of the liver and heart of the hyperthyroid animals.

Summary. Young hyperthyroid rats fed a diet high in crystalline B-complex vitamins grew fairly well but nevertheless showed a low concentration of glycogen in their livers and hearts. Additional supplementation with liver meal increased their growth rate to normal, but exerted no apparent favorable effect on the glycogen content of these organs. The decrease in cardiac glycogen concentration can be explained in part on the marked cardiac hypertrophy which is associated with experimental hyperthyroidism in the rat.

6. Wesson, L. G., *Science,* 1932, v75, 339.

7. Goode, C. A., Kramer, H., and Somogyi, M., *J. Biol. Chem.*, 1933, v100, 485.

TABLE II.
Effects of Dietary Supplements on Concentration of Glycogen in Liver and Heart of Hyperthyroid Rats

Exp. No.	No. of rats	Avg daily gain, g	Avg liver wt, g	Avg liver glycogen, %	Heart % body wt	Heart glycogen %	Heart glycogen Mg per 100 g body wt	Diet used
8	7	.16	3.9	.6 (.01-3.1)	.62	—	—	Diet 1 + .13% thyroid.
	6	.42	4.9	.4 (.01-0.8)	.64	—	—	As above + 2 mg biotin + 5 mg folic acid/kg.
	8	1.9	7.5	.5 (.01-0.6)	.63	—	—	Diet 2 + .13% thyroid.
	6	2.4	7.1	5.7 (4.1-8.4)	.34	—	—	Diet 1
14	7	1.7	6.6	.2 (.02-0.8)	.61	.11	.49	Diet 2 + .13% thyroid.
	8	1.7	6.5	.04 (.01-0.1)	.76	.08	.51	As above + 5 mg folic acid/kg.
	6	2.2	7.8	.04 (.01-0.1)	.64	.05	.40	As above + 5 mg folic acid + 2 mg biotin/kg.
	10	3.1	6.4	1.9 (.06-4.6)	.33	.46	1.56	Diet 2
34	6	2.9	9.5	1.9 (1.2-3.4)	.55	.06	.33	Diet 2 + 10% liver meal* + .25% thyroid.
	6	2.6	9.4	1.7 (.2-2.3)	.57	.12	.65	Diet 2 + 10% liver meal† + .25% thyroid.
	5	2.9	7.0	2.1 (.2-7.5)	.61	.06	.50	Diet 2 + .25% thyroid.
	10	3.0	5.0	7.0 (3.0-9.8)	.37	.24	.93	Diet 2.
40	5	3.2	10.0	.9 (.04-1.7)	.50	.16	.78	Diet 5 + .4% thyroid.
	4	2.3	7.8	.2 (.01-0.6)	.64	.11	.52	Diet 4 + .4% thyroid.
	5	3.2	8.3	5.5 (2.3-7.9)	.32	.26	.76	Diet 4.
39	8	2.8	5.2	.07 (.05-0.1)	.52	.11	.67	Diet 3 + 30 γ B_{12}/kg + .25% thyroid.
	4	2.9	5.9	.07 (.04-0.1)	.60	.11	.67	Diet 3 + .25% thyroid.

* Dried water insoluble residue from the manufacture of the anti-P.A. factor.
† Viobin, defatted liver.

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Progestational Activity of 1,2-*bis*-(p-Aminophenyl)-2-Methylpropanone-1 Dihydrochloride. (17999)

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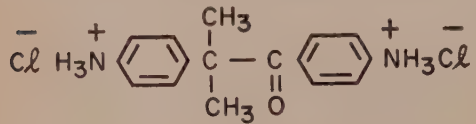
In the course of a comprehensive study of the relationship between chemical structure and biological activity in a series of substituted desoxybenzoins it was found that 1,2-bis-(p-aminophenyl)-2-methylpropanone-1 dihydrochloride exhibits progestational activity.



COMPOUND I.

The synthesis and properties of this compound have been previously described (Allen and Corwin)(1). We have employed a modification of the biological test for progesterone described by Clauberg(2). Juvenile female rabbits (New Zealand Whites) initially weighing between 600 and 800 g were utilized. Each test animal was pre-treated daily for 6

1. Allen and Corwin, *J.A.C.S.*, 1950, v72, 117.
2. Clauberg, C., *Zentralblatt f. Gynak*, 1930, v54, 2757

TABLE I.
Progestational Effect of 1,2-*bis*-(p-aminophenyl)-2-methylpropanone-1 Dihydrochloride.

Compound injected	Daily dose, mg	Vehicle and vol., cc	Uterine wt., mg	Body wt	Grade of response
I	166	H_2O/2	1828	1068	+2
I	166	"	1435	1109	+1
P	0.5	0.1 oil	2660	1152	+4
I	332	H_2O/4	1420	1265	+4
I	332	"	2900	1002	+3
I	166	H_2O/2	2010	1318	±
I	166	"	1420	1120	+1
I	332	oil/4	2014	1300	+2
I	332	"	2000	1140	+2
P	0.5	.1 oil	6660	1322	+4
P	0.5	"	4900	1000	+4
I	200	H_2O/2	2410	1331	+3
I	200	"	2100	1141	+4
I	400	"	4335	1110	+4
I	400	"	3780	982	+4
I	600	"	3115	1232	+3
I	600	"	2115	1249	+4
I	100	H_2O/2	4340	1700	+3
I	100	"	3930	1822	+3
I	200	H_2O/4	4590	1265	+4
I	200	"	4450	1650	+2
I	400	H_2O/8	3770	1834	+4
I	400	"	4370	1539	+4
I	600	H_2O/12	4250	1532	+4
I	600	"	5730	1314	+4
P	0.5	.5 oil	10140	1655	+4
P	0.5	"	8220	1734	+4
I	50	H_2O/1	2035	1150	±
I	50	"	1230	1162	±
I	100	H_2O/2	2030	1160	±
I	100	"	1830	1076	±
P	.25	.25/oil	3525	1008	+4
P	.25	"	2200	968	+4

* I = 1,2-*bis*-(p-aminophenyl)-2-methylpropanone-1 dihydrochloride.
P = Crystalline progesterone.

days with 10 μg of estradiol benzoate administered subcutaneously in 0.5 cc corn oil. For the ensuing 5 days either Compound I or progesterone was administered subcutaneously in the dosage and form indicated in Table I. Twenty-four hours after the last injection the animals were autopsied, their terminal body weight and uterine weight recorded, and the uterus prepared for histological grading of the progestational response as described by Hisaw and Leonard(3).

The data indicate that of 24 rabbits given a daily dose of 100 mg or more, all but 2 showed a decisive progestational response to Compound I. The grade of response varied considerably but such variability is characteristic of the progestational response in the young rabbit(2). In 2 rabbits treated with 50 mg daily only a doubtful response was elicited and a similarly marginal effect was seen in 2 rabbits receiving 100 mg daily. By comparison with the potency of progesterone under similar experimental conditions, it may be crudely estimated that Compound I is about 0.5 to 0.25% as active as progesterone.

From the observations on uterine weight it is apparent that Compound I brings about less increment in uterine weight than progesterone even in instances in which the progesta-

3. Hisaw, F. L. and Leonard, S. L., *Am. J. Phys.*, 1930, v92, 574.

tional effect upon the endometrium is maximal.

The progestational property of progesterone has been considered highly specific in that it is known to be possessed by only few closely related steroids and has not been previously demonstrated in non-steroidal compounds. Desoxycorticosterone and 17-hydroxy-11-dehydrocorticosterone (Cpd. E. of Kendall) are known to possess limited degrees of progestational activity(4). Conversely, progesterone has a limited corticoid effect(4). In view of the progestational activity of Compound I, the potential corticoid activity of this and related substances adds interest to the further biological characterization of this series of compounds. We shall subsequently report on the chemistry and physiological activities of other substituted desoxybenzoins.

4. Selye, H., Encyclopedia of Endocrinology, Section I, Steroids, I-IV; Richardson, Bond and Wright Co., Montreal, Canada, 1943.

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Effects of Radioactive Iodine on Free Sarcoma 37 Cells in the Peritoneal Fluid of the Mouse.* (18000)

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In producing biological effects on living tissue cells, the gamma rays of radioactive isotopes expend their energy by ejection of high speed electrons. Consequently, it was presumed that the action of beta particles on cell complexes would be based on similar mechanism. However, it is known that beta particles do not penetrate significantly in the body or organ surface exposed to irradiation (Dobson and Lawrence)(1). Moreover it is difficult to distinguish the primary effect of beta particles on tumor cells from the secondary effect induced by the irradiated layer of malignant tissue in tumor stroma, blood vessels and deeper portions of the tumor. It seems reasonable to study the primary effect of beta particles on tumor cells, by introducing radioisotopes in body fluids containing free tumor cells. Thus, Evans and Quimby(2) injected radioactive sodium subcutaneously into leukemic mice and recorded the effects on blood counts. We have found that it is convenient to induce and to observe primary changes in tumor cells by injecting a radioactive isotope intraperitoneally into mice bearing viable free tumor cells in their peritoneal fluid. The viability of such cells has been demonstrated by several authors: by Graham(3) in human patients, by Warren and Gates(4) in rats and by several German authors(5) in mice. Recent experiments (Goldie and Felix)(6) have shown that it is possible to grow in the peritoneal fluid free tumor cells (sarcoma 37 cells in CFW mice, thymoma cells in dba mice) in serial intraperitoneal transfers. At each transfer the multiplication of tumor cells can be estimated quantitatively by counting cells and their mitoses first in the inoculated fluid and later on in specimens of peritoneal fluid from inoculated mice. This method was applied in the experiments reported below recording the changes in number and characteristics of free sarcoma 37 cells after intraperitoneal injection of radioactive iodine.

* This work was carried out under Contract AT-(40-1)-269 with the Division of Biology and Medicine, U. S. Atomic Energy Commission.

1. Dobson, R. L., and Lawrence, J. H., *Ann. Rev. Physiol.*, 1948, v10, 479.

2. Evans, T. C. and Quimby, E. H., *Am. J. Roentgenol. and Radium Ther.*, 1946, v55, 55.

3. Graham, G. S., *Am. J. Path.*, 1933, v9, 701.

4. Warren, Sh. and Gates, O., *Am. J. Cancer*, 1936, v27, 485.

5. Seeger, P. G., *Arch. Exp. Zellforsch.*, 1937, v20, 280.

6. Goldie, H. and Felix, M., to be published.

Technic. (1) *Inoculation of tumor cells.* The number of tumor cells in the peritoneal fluid of a "donor" mouse was calculated by counting the total number of cells (tumor cells, leucocytes, peritoneal tissue macrophages) in the fluid (using an hemocytometer) and the percentage of tumor cells (differential counts in the smears stained with Wright's stain and by Aceto-Orcein method) in the same specimen. The requisite number of tumor cells was obtained by diluting the peritoneal fluid to the concentration of 10,000 cells (standard dose) or 50,000 cells (maximum dose) in 0.5 cc. The needle was thrust under the skin at the junction of the middle and the lower third of the abdominal axillary line and the peritoneum was pierced ½ inch lower (to avoid leakage). The mice used in the experiment were CFW strain of Carworth Farms. The tumor was a sarcoma 37 strain maintained in more than 70 intraperitoneal transfers.

(2) *Treatment.* Radioactive iodine (I^{131})† was injected intraperitoneally in doses of 0.2 to 0.4 mc, 2 or 3 days after inoculation of tumor cells by the same route. This interval of time was fixed after observation that late treatment (more than 3 days after inoculation) does not prevent the appearance of subcutaneous tumors at the site where the inoculating needle was thrust through the skin. The localization of free tumor cells in the connective tissue at the site of trauma by injection or exploratory puncture of the abdomen occurred, within 4-6 days, in all control mice, inoculated and untreated. Twenty-five control mice were treated with inactive iodine, *i.e.*, a specimen of I^{131} preparation which lost its activity. All animals survived at least 7 days after administration of the maximal therapeutic dose of iodine used in these experiments (0.4 mc, about 0.02 mc per 1 g weight).

(3) *Assays of the effect on tumor cells.* Amounts of 0.1 or 0.2 cc of peritoneal fluid were withdrawn from inoculated mice 1, 2 or 3 days after treatment, in some instances after 4, 7 or 10 days. In control mice the fluid was withdrawn at similar intervals after inoculation. The withdrawn material was used for microscopic assay (presence of tumor cells in the fluid) and for biological assay (viability of remaining tumor cells). (a) *Microscopic assay: morphological changes in tumor cells or their disappearance from the fluid.* A small drop (about 0.01 cc) of each specimen was smeared thinly on each of two slides: one was stained with Wright's stain (for differential study of tumor cells and leucocytes) and the other with Aceto-Orcein (for counting of mitoses). The results were compared with those in specimens from control mice. Disappearance of mitoses and eventually disappearance of tumor cells were considered as marking points in each experiment. (b) *Biological test: viability of tumor cells from treated mice after their i.p. transfer into normal mice.* Major portions of the fluid (0.05 to 0.2 cc) withdrawn at regular intervals after treatment were transferred intraperitoneally into new mice, and in these mice the fluid was examined by repeated withdrawals as above, but the period of observation lasted up to 20 days. If the microscopic test revealed the presence of mitotic tumor cells in the fluid of recipients, this fluid was transferred (2nd transfer) into new mice, from these eventually (3rd transfer) into new mice and so on. The number of transfers during which the tumor cells maintained their morphological integrity and ability to multiply (mitoses) was considered to be an index of their viability. (c) *Test for radioactivity.* The disappearance of injected radioactive preparation from the peritoneal fluid was checked by testing with a Geiger counter.

(3) *Results.* (a) *Tumor cells in the peritoneal fluid of control mice.* In more than 100 control mice (untreated or treated with decayed I^{131}) the inoculated 10,000 tumor cells multiplied, after a lag period of a few hours, and reached within 4 to 6 days the concentration of 40,000 to 60,000 per cc (the total amount of fluid in the peritoneal cavity varied approximately from 0.2 to 0.5 cc). The percentage of mitoses in these cells was of 15 to 25%. (Fig. 1). After the 6th or the

† Carrier-free I^{131} was obtained from the Isotopes Division, U.S.A.E.C., Oak Ridge, Tenn.

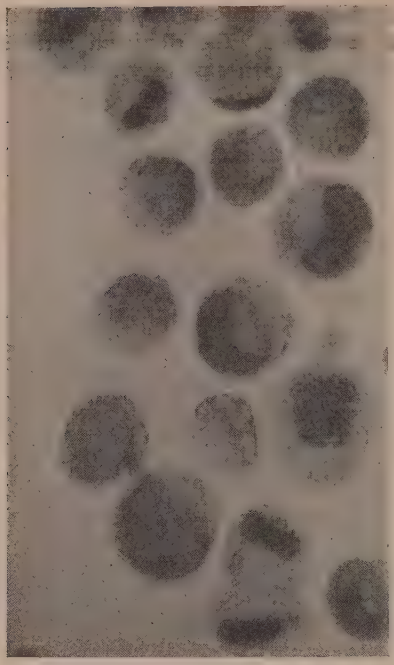

FIG. 1.
Sarcoma cells in the peritoneal fluid of the mouse 5 days after i.p. inoculation of 10,000 sarcoma cells. Aceto-Orcein ×1000.

7th day the number of intact tumor cells and the percentage of mitoses decreased slowly, while the percentage of necrotic cells increased as long as the mice survived. All animals developed solid implants at the site of intraperitoneal inoculation and died mostly within two weeks. The intraperitoneal transfer of the peritoneal fluid from the control mice into new mice resulted always in tumor cell multiplication and later on in growth of solid tumor at the site of inoculation. The subcutaneous inoculation of this fluid induced solid tumors. The serial peritoneal transfers of peritoneal fluid from control mice could be continued indefinitely without any decrease in the extent of tumor cell multiplication at each transfer.

(b) *Tumor cells in treated mice.* Twenty-four hours after treatment, no tumor cells were found in 9 out of 34 mice treated with 0.2 to 0.22 mc of I^{131}, (1st group) and in 51 out of 72 mice treated with 0.4 to 0.44 mc of the same preparation (2nd group). In remaining mice, some cells were apparently normal and showed mitoses, but many cells were distinctly altered; their cytoplasm was large and vacuolized, the chromatin separated in fragments. In apparently intact cells, the proportion of mitoses was quite high (10 to 20%), but within the next 2 days, it dropped to less than 5% and in most mice to less than 2%. The chromosomes in these mitoses were atypically arranged or abnormal (Fig. 2). The majority of surviving tumor cells appeared markedly changed on the 3rd day or the 4th day after treatment. Thus, the damage inflicted to tumor cells by the treatment was progressing for several days after the injection of I^{131}. As the final result, only 2 out of 106 treated mice showed in their peritoneal fluid mitotic tumor cells on the 8th day after treatment. This microscopic evidence of damage to tumor cells was strongly corroborated by the evidence of biological tests: Tumor cells appearing intact or con-

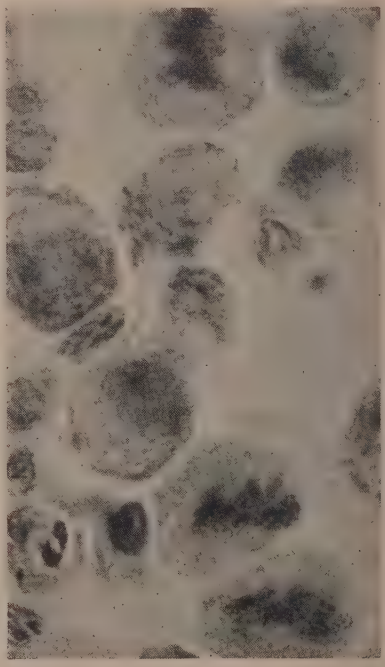

FIG. 2.
Sarcoma cells in the peritoneal fluid of the mouse, 5 days after i.p. inoculation of 10,000 sarcoma cells, 48 hours after i.p. injection of radioactive iodine (0.2 mc). Aceto-Orcein ×1000.

taining mitoses were transferred with the peritoneal fluid of 30 mice of the first group and 30 mice of the second group into 60 new mice; only 7 recipients showed the occurrence of tumor cells and mitoses in their peritoneal fluid after transfer, while in 53 recipients the transferred cells died more or less rapidly. The "second transfer" of fluid using seven positive recipients as donors resulted in cell multiplication in only one mouse. None of 106 treated mice developed a small nodule. Subcutaneous inoculation of 0.2 cc of fluid from 30 treated mice failed to induce tumors or even nodules in normal mice. Only one tumor strain isolated from a treated mouse (mentioned above) was able to induce regularly subcutaneous tumors. This strain (which we have designated as strain C) showed higher growth potency than the original sarcoma strain, it grew more vigorously (in 25 transfers up to the present time) and induced larger amounts of fluid and only a slight leucocytic reaction at each transfer. Two groups each of 12 mice were inoculated with 50,000 tumor cells and treated with 0.2 (1st group) or 0.4 mc (2nd group) of I^{131}. The tumor cells disappeared within 3 days in 9 mice of the first group and in 4 mice of the second group. In 9 out of 11 "positive" mice, the tumor cells disappeared after the first transfer and in remaining 2, after the second transfer.

(c) *Radioactivity of the peritoneal fluid.* The peritoneal fluid of all mice injected with I^{131} showed weak radioactivity at the end of the first 24 hours, but only in few mice at the end of the second day. In no instance, did the fluid remain radioactive at the end of the third day. After transfer of treated cells into new mice, the peritoneal fluid of recipients consisted in 70 to 80% of large macrophages vacuolized and stuffed with debris of tumor cells.

Discussion. Immediate morphological changes and, in several mice, rapid disappearance of tumor cells from the peritoneal fluid were observed after treatment with radioactive I^{131}, but not after injection of decayed I^{131}. Thus, the biological effect of I^{131} on tumor cells should be attributed to its radioactivity. The radioactivity test of the peritoneal fluid indicated that the injection preparation was immediately dissolved in the peritoneal fluid and distributed even in the "pockets" of the peritoneal cavity. Thus, the tumor cells, the leucocytes and the macrophages were suspended in a radioactive fluid medium. In these conditions the chief effect on the cells would likely be produced only by beta particles of the preparation. The radioactivity of the peritoneal fluid did not last more than 24 to 48 hours, while the signs of damage to cells continued to increase after that period. Thus, the primary action of beta particles was a "hit and run" blow to tumor cells, but it was followed by secondary effect of damage to these cells. The decrease in the viability of tumor cells was manifested by their inability or limited ability to multiply into serial intraperitoneal transfers and to induce sizable subcutaneous tumors. Since Lea(7) interpreted the killing of bacteria by radiation as a lethal mutation, it may be presumed that similar phenomenon is responsible for the loss of viability of irradiated tumor cells in the peritoneal fluid. The mutant tumor cell loses its essential characteristic of autonomic growth and therefore lacks the ability to survive and multiply after transfer into a new host. In the case of tumor strain C the tumor cells from treated mice continued to grow vigorously in serial transfers. This may be attributed to survival of particularly "tough" tumor cells after destruction of all "weak" individuals by radiation or to a mutation resulting in better adjustment of tumor cells to the host.

Summary and conclusion. 1. CFW mice of 25 g weight were inoculated intraperitoneally with requisite numbers (mostly 10,000) of S-37 cells and treated *i.p.* with radioactive iodine (I^{131}) (0.2 or 0.4 mc). The fate of tumor cells was studied by repeated withdrawal and examination of peritoneal fluid in each mouse and compared with their fate in control animals (inoculated with the same dose of cells, but untreated or treated with decayed I^{131}).

7. Lea, D. E., Actions of Radiation on Living Cells, N. Y., Macmillan, 1947, Chap. 9.

2. The examination of each specimen of peritoneal fluid consisted (a) in the microscopic assay of integrity and ability for multiplication (percentage of mitoses) of free tumor cells which multiplied in the peritoneal fluid before treatment and (b) in the biological assay of viability of tumor cells, *i.e.*, their ability to survive and multiply after transfer into new mice.

3. The results indicate that with a single exception (tumor strain C isolated from a treated mouse) all tumor cells suffered loss of viability even after treatment with the minimal dose (0.2 mc) of I^{131}. In most mice this effect was detected only after the failure of tumor cells from treated animals to grow in the peritoneal fluid of new animals. In other animals, where higher dose of radiation was used or, presumably, where nutrient environment was drastically changed, the tumor cells disintegrated in the body of the treated mouse.

4. Rapid disappearance (after 24 to 48 hours) from the peritoneal fluid of injected radioactive preparation suggested the differentiation between its early and primary effect (which may be the production of a "lethal mutation") and its later secondary effect (cell disintegration) may be due to additional factors (higher dose of radioactivity, drastic changes in the biological medium).

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Distribution and Effect of Colloidal Radioactive Gold in Peritoneal Fluid Containing Free Sarcoma 37 Cells.* (18001)

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Hahn and Sheppard(1-3) introduced the use of colloidal radioactive isotopes by intravenous administration in the treatment of diseases of the lymphoid macrophage system. Subsequently, they and their colleagues suggested the use of such colloids, especially that of the insoluble radioactive metallic gold sols, in the treatment of malignant tumors by direct infiltration(4). Administered by the intravenous route these particles are rapidly removed from the circulation through phagocytosis by the reticulo-endothelial cells of the liver and spleen, and to a much less extent by the bone marrow and lymph nodes (5). Given by the direct infiltration route, these particles, insoluble in body fluids, remain for the most part *in situ* except for a certain degree of drainage through the fascial planes and through the neighboring lymphatics. Thus, a certain degree of selectivity may be effected through the direction of the operator in the treatment of certain malignant disease processes. Distribution studies by Hahn, Sheppard and their associates(5) in humans and dogs, by Barrow and associates (6) in rabbits, and by Sheppard and Furth (7) in mice showed that intravenously injected gold colloids are stored in the organs rich in the reticulo-endothelial elements

* This work was carried out under Contract AT-(40-1)269 with the Division of Biology and Medicine, U. S. Atomic Energy Commission.

1. Hahn, P. F. and Sheppard, C. W., *So. Med. J.*, 1946, v39, 558.

2. Sheppard, C. W. and Hahn, P. F., *So. Med. J.*, 1946, v39, 562.

3. Sheppard, C. W., Goodell, J. P. B. and Hahn, P. F., *J. Lab. and Clin. Med.*, 1947, v32, 1437.

4. Hahn, P. F., Goodell, J. P. B., Sheppard, C. W., Cannon, R. O., and Francis, H. C., *J. Lab. and Clin. Med.*, 1947, v32, 1442.

5. Sheppard, C. W., Wells, E. B., Hahn, P. F. and Goodell, J. P. B., *J. Lab. and Clin. Med.*, 1947, v32, 274.

6. Barrow, J., Tullis, J. L. and Chambers, F. F., *Nav. Med. Rev. Inst.*, Bethesda, Md., Prog. AM007 039, rep. No. 24, July 25, 1949.

7. Sheppard, C. W., Furth, J. and Wish, L., *Fed. Proc.*, 1950, v9, 343.

(macrophages) mainly in the liver and spleen. The radioactive colloidal gold used in these studies was obtained by bombardment by slow neutrons in the chain reacting pile, the target material being 100% naturally abundant Au^{197}. The cross section of the latter to slow neutrons affords a highly economical end product for therapeutic use and the colloid(3) is extremely stable and meets very satisfactorily the criteria set up for therapeutic use of radioactive isotopes(8). The colloid as used contains about 4 mg of gold per cc.† In mice inoculated intraperitoneally with S-37 cells, the peritoneal exudate contains numerous, rapidly proliferating tumor cells, as well as leucocytes and macrophages (9). Thus, it could be used conveniently to study simultaneously the effect on tumor cells and the fate in macrophages of the colloidal gold injected intraperitoneally. The essential results of this study are reported below.

Technic. Our methods of inoculation of tumor cells and of assays of the results were described previously(9). A single intraperitoneal injection of gold containing Au^{198} was performed in inoculated mice. Doses of 0.2 to 0.42 mc were injected in a volume of 0.5 cc. Thus, the original preparation was diluted, according to its activity, 10 to 20 times. The control mice remained untreated or received various doses of inactive colloidal gold, *i.e.*, a preparation formerly containing Au^{198} stored several months to allow decay of its radioactivity. Some mice received as much as 0.25 cc of undiluted inactive gold. Following tests for integrity of macrophages and polymorphonuclears were used: (a) Diluted (1:10) mouse blood was injected (0.5 cc) in the peritoneal cavity of treated mice and controls; occurrence or absence of phagocytosis of red blood cells was recorded. Similar technic was used to test the phagocytosis of bacteria by polymorphonuclears. (b) Alcoholic 0.4% solution of neutral red (few drops) was evaporated on a slide, and a drop of peritoneal fluid was placed on stained area and covered with a cover glass; within 5 minutes all necrotic elements were diffusely stained; cells with only stained inclusions were considered as undamaged.

Results. (1) *Survival of mice.* Out of 48 mice inoculated *i.p.* with about 10,000 S-37 cells and treated, 2 or 3 days later, by the same route with 0.4 mc of gold only 15 survived less than 7 days. Out of 144 mice treated 2 to 5 days after inoculation with 0.2 to 0.33 mc of gold, 54 died or were sacrificed within the first 7 days, and the remaining animals survived at least 14 days (*i.e.* as long or longer than the control animals).

(2) *Effect on tumor cells.* In all 136 mice treated at the end of the 48 hour period after inoculation and in 19 out of 24 mice treated after 72 hours, only scarce and cytologically abnormal tumor cells were found in the peritoneal fluid examined 24 hours after

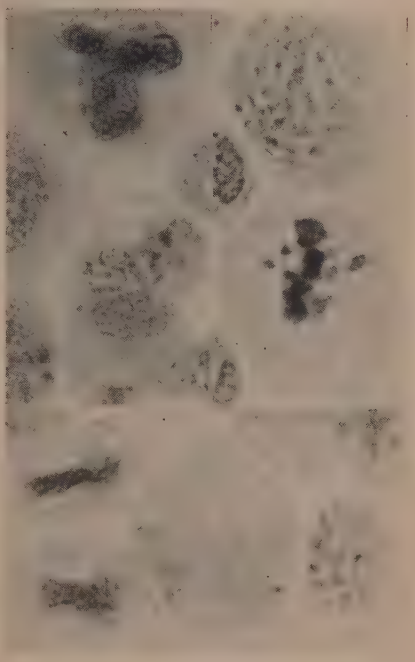

FIG. 1.
Sarcoma cells in the peritoneal fluid of the mouse, 6 days after i.p. inoculation of 10,000 sarcoma cells and 24 hr after i.p. inj. of colloidal radioactive gold (0.33 mc). Aceto-Orcein ×1000.

8. Hahn, P. F., A Manual of Artificial Radioisotope Therapy, Chapter III, Academic Press, New York, 1950.

† Colloidal radioactive gold containing Au^{198} was obtained from Abbott Laboratories, North Chicago, Ill.

9. Goldie, H. and Hahn, P. F., PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 634.

treatment and at any later date. However, in 5 out of 24 mice treated on the 4th day and in all 32 mice treated (with 0.2 to 0.4 mc of gold) on the 6th day, many tumor cells were present in the fluid withdrawn 24 to 48 hours after treatment. These cells were mostly different from those in controls; in resting cells their cytoplasm was unusually large and the nucleus contained higher number of nucleoli; in dividing cells the chromosomes were asymmetrically arranged, often overcondensed in large clumps; they formed bridges between opposite poles and frequently showed delayed division (stickiness) in telophase and prophase (Fig. 1), as well as giant cells with small chromosomes or with 2 nuclei. In order to test the viability of these cells, they were transferred in 0.1 to 0.2 cc of peritoneal fluid from each of 30 mice *i.p.* into 30 new mice and subcutaneously into 15 mice. Repeated withdrawal of the fluid from the recipients did not reveal any growth or even survival of transferred cells, and no subcutaneous tumors were recorded. Moreover, the specimens of the peritoneal fluid obtained from the "donors" on the 3rd day after treatment contained only a few abnormal tumor cells or their debris and numerous macrophages. Twelve mice were inoculated with a higher number of S-37 cells (50,000) and treated, after 2 days, with 0.4 mc of gold; in all mice the tumor cells disappeared from the peritoneal fluid or became very scarce within 24 hours. In control mice, the inoculated sarcoma cells multiplied vigorously in the fluid (15 to 25% mitoses) until the 6th or 7th day; at that date the percentage of their mitoses began to decrease and dropped to a low level (about 5%) on the 10th day in the surviving mice. In all controls intraperitoneal and subcutaneous implants were found about the 7th day at the site of inoculation. The *i.p.* transfer of the peritoneal fluid from these mice, at any stage after inoculation, induced in the recipient the same growth cycle of tumor cells.

(3) *The presence of gold particles in macrophages.* The gold colloids used in these experiments were exceedingly highly dispersed with ultramicroscopic particles ranging in size from about 10 to 50 millimicra. Immediately following injection one would not expect to find microscopically visible particles in any tissues examined(5). Thus, the agglomeration noted below is of considerable interest. The intraperitoneal injection of colloidal gold preparations in amounts as small as those used in our experiments (0.08 to 0.15 mg) was followed after 24 to 48 hours (in an experiment with one specimen of the preparation within 18 hours) by the appearance of dot-like gold particles in the macrophages (Fig. 2). In this stage, these groups of spherical dots resembled a swarm of cocci, keeping closely together even when the cytoplasm was not outlined. If it were sharply outlined some particles were always found apparently attached to the outer side of the cell membrane. On the following days the gold dots were found replaced by clumps of irregular spherical shape, often adjacent to the cell membrane, until finally only one or 2 large black clumps were found in each macrophage. In control animals injected with similar amounts of inactive colloidal gold, the intracellular formation and aggregation of coarse gold particles inside the macrophages or on their membranes followed the same pattern, but the clumps showed the tendency to remain dispersed, without final

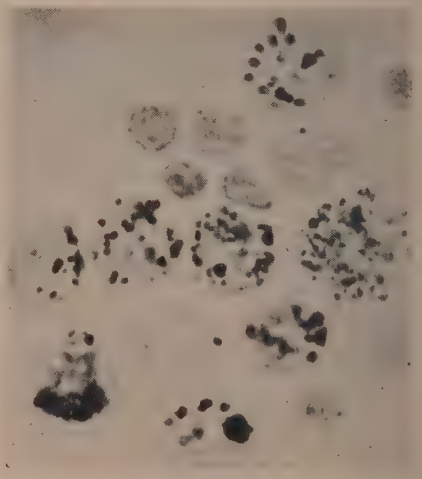

FIG. 2.

Macrophages in the peritoneal fluid of the mouse, 5 days after i.p. inoculation of 10,000 sarcoma cells and 3 days after i.p. inj. of colloidal radioactive gold (0.33 mc). Aceto-Orcein ×1000.

formation of few single clumps.

The neutral red test and the phagocytosis test revealed in samples of peritoneal fluid withdrawn 2 or 3 days after treatment that the majority of free macrophages loaded with small or large clumps of colloidal radioactive gold showed tinctorial characteristics of undamaged cells and maintained their phagocytic function. In the same samples of fluid (containing radioactive gold) many polymorphonuclears were able to absorb bacteria added to the fluid. Peritoneal fluid showed rapidly decreasing radioactivity during 3 days following the injection (only traces on the 4th day). This activity was concentrated in the cellular sediment of the peritoneal fluid and present only in traces in supernatant fluid.

Discussion. A full radiation effect on tumor cells was obtained by using the peritoneal fluid as growth environment for these cells, and colloidal gold containing Au^{198} as source of radioactivity. Since the half-path of the beta particles is about 0.4 mm in tissue, while for most gamma rays it is much longer, it may be presumed that the ionizing radiation responsible for radiation effect was derived to an extent of about 90% from the beta particles of gold suspension intimately mixed with tumor cells in the peritoneal fluid(10). The destruction of tumor cells was evidenced(1) by their advanced stage of distintegration or their absence in stained smears from peritoneal fluid; (2) by the absence of subcutaneous or intraperitoneal implants in treated mice at the site of inoculation and (3) by the lack of viability of treated cells, as shown by their inability to grow after *i.p.* or *s.c.* transfer into new mice. This marked radio-therapeutic effect of colloidal radioactive gold on peritoneal tumor cells in mice corresponds to recent clinical observations of Müller(11) in 8 female patients with carcinomatous peritonitis where it was claimed that the intraperitoneal administration of colloidal radioactive gold (100 to 150 mc) induced "undoubtful therapeutic results".

The cytological abnormalities observed in tumor cells as a result of radiation resembled closely those recorded by other authors and reviewed recently by Koller(12): fragmentation or over-condensation of chromosomes, prolongation of metaphase and of late prophase stages, lagging of chromosomes and increased number of nucleoli in resting cells.

The absence of microscopically visible gold dots or clumps in free cells other than macrophages suggests that ultramicroscopic gold particles are selectively absorbed by macrophages and do not simply adhere to any cell surface. On the contrary, the agglomeration into larger clumps may be purely physical phenomenon, analogous to those observed in several colloidal systems under similar physicochemical conditions. The tendency of inactive colloidal gold to remain dispersed at a certain level may be due to the difference in electric charges of radioactive and inactive gold particles.

Our demonstration of morphological and functional integrity of macrophages loaded with radioactive gold clumps indicates the remarkably high resistance of these cells to intracellular radiation effects. The absence or scarcity of floating free gold clumps in the fluid, even after transfer of gold bearing macrophages into the peritoneal cavity of new mice, suggests that the gold clumps from macrophages which eventually disintegrate are immediately absorbed or adsorbed by the tissue macrophages in the peritoneal wall. This is supported by extreme abundance (macroscopic grey patches) of gold clumps in the peritoneal tissue macrophages. Our data indicate that many polymorphonuclears in the peritoneal fluid were destroyed (or their life cycle was shortened) by the radiation effect, but those which escaped this effect maintained their functional integrity as shown by phagocytosis of bacteria.

Summary and conclusions. 1. Two to 5 days after intraperitoneal inoculation of 10,000 (in one series—50,000) free sarcoma-37 cells from peritoneal fluid, several series of CFW mice were treated with a single intra-

10. Peacock, W. C., personal communication.

11. Müller, J. H., *Bull. Schweiz, Akad. d. Med. Wissensch,* 1949, v5, 484.

12. Koller, P. C., *Brit. J. Cancer,* 1947, v1, 38; and Koller, P. C. The Behavior of Tumor Cells Under Normal and Experimental Conditions. Symposium on Biol. Science, Univ. of Milan, 1949.

peritoneal injection of 0.2 to 0.44 mc of colloidal radioactive gold. Control mice remained untreated or were treated with inactive (decayed) gold preparations.

2. On 3 consecutive days after treatment, specimens of peritoneal fluid were withdrawn by exploratory puncture from treated and control mice. Stained smears from these specimens revealed the absence of tumor cells or cytological abnormalities therein in gold treated mice, while in controls the tumor cells were numerous and showed high proportion of mitoses. The non-viability of morphologically alterated cells was demonstrated by their inability to multiply or even to survive after their transfer into new mice.

3. Ultramicroscopic colloidal gold particles mixed immediately after injection with the peritoneal fluid became condensed, after 24 to 48 hours, into dot-like particles in the cytoplasm of macrophages and during the following days into coarse clumps. The macrophages loaded with gold particles maintained their structural and functional integrity; lymphocytes and polymorphonuclears were partially and temporarily destroyed by radiation effect.

4. It is concluded that in the cellular peritoneal exudate from mice inoculated *i.p.* with S-37 cells, the tumor cells were highly sensitive to small amounts of radiation well tolerated by the mice themselves while macrophages showed very high resistance to the same agent. Thus, the complete destruction of free tumor cells in the peritoneal cavity of the mice may be described as a selective radiotherapeutic effect of radioactive colloidal gold.

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A Simple Chromatographic Procedure for the Separation of Angiotonin from Crude Mixtures. (18002)

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The purification of angiotonin (hypertensin) has been hampered not only by its lability but by its relatively high solubility in water and low solubility in ordinary organic solvents. In preliminary chromatographic experiments with the capillary-ascent test tube method of Rockland and Dunn(1), using 7% aqueous solution of phenol as the developing agent, it was found that angiotonin could be separated from materials that gave a positive ninhydrin color reaction. Although a satisfactory separation could be obtained with small quantities by this technic, as much as 800 to 4000 units of angiotonin could be isolated on a column prepared from No. 1 Whatman paper macerated in a Waring blendor with 7% aqueous phenol solution.

Methods. Preparation of column. Whatman No. 1 filter paper was macerated in a Waring blendor with 5% aqueous phenol (7 g reagent grade phenol in 100 cc distilled water) and transferred to a sulfur absorption tube of 30 mm diameter (Corning 39620) the fritted glass disc of which had been covered with a layer of glass beads. The tube was fitted into a suction flask by means of a one-hole rubber stopper. Suction was applied to the tube while the pulped paper was added in small portions, a wide-footed glass rod being used to aid in forming a tightly packed column. The column was washed with 7% phenol solution to remove any material possibly soluble in the solution.

Preparation of angiotonin. The angiotonin was prepared by Plentl and Page's modification(2) of the method of Page and Helmer(3) from renin substrate processed from hog serum and an angiotonase-free hog renin.

1. Rockland, L. B., and Dunn, M. S., *Science*, 1949, v109, 539.

2. Plentl, A. A., and Page, I. H., *J. Biol. Chem.*, 1945, v158, 49.

3. Page, I. H., and Helmer, O. M., *J. Exp. Med.*, 1940, v71, 29.

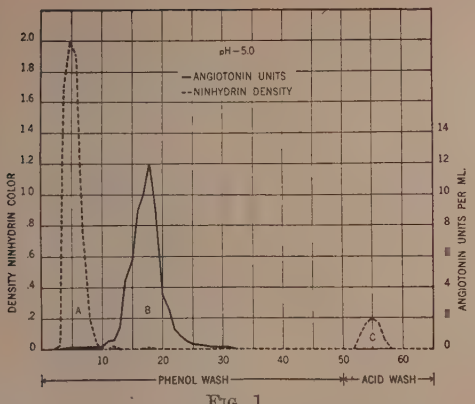

FIG. 1.

Chromatogram of a crude angiotonin preparation at pH 5.0. The number of 10 cc aliquots of effluent are shown by the abscissa.

Angiotonin assay. The angiotonin was tested in pithed cats prepared as described by Shipley, Helmer, and Kohlstaedt(4). The various samples were compared to a lyophilized laboratory standard of angiotonin, 30 μg of which is designated as 1 unit.

Ninhydrin color reaction. The photometric ninhydrin method of Moore and Stein(5) was used to determine the amount of ninhydrin color produced in the aliquots of effluent. The values were expressed in density units per ml of effluent.

Procedure. The crude angiotonin preparations to be fractionated were adjusted in different experiments at pH 4.6 to 7.0. In Fig. 1 is shown an experiment at pH 5.0 in which the paper column was 70 mm in height. Ten ml of angiotonin containing 80 units per ml were poured onto the top of the paper. Then 1 ml of 80% phenol solution was added to bring the mixture to a concentration of approximately 7% phenol. Gentle suction was applied to pull the solution into the paper. The column was then washed with 7% phenol until 50 aliquots of 10 ml of effluent had been collected. Then 0.1% acetic acid was substituted for the phenol solution and another 15 samples were obtained. Each aliquot was extracted with ether to remove phenol and assayed for pressor activity and ninhydrin reacting components.

Results. As can be seen from Fig. 1, there was a fairly sharp separation of angiotonin from the components giving a positive ninhydrin reaction. There was also considerable purification. The starting material required 765 μg to give a pressor response equivalent to 1 angiotonin unit, whereas 38 μg of fraction B were equal to 1 unit. Consequently one passage through the column produced a 20-fold purification. The recovery from such experiments before lyophilization has been from 79 to 98%. Fractions A and C were without significant pressor activity when injected in quantities of 200 μg. Sample A, given in greater concentration, did have pressor properties, but, as demonstrated by its resistance to angiotonase, the pressor activity was not due to angiotonin. Of the pH adjustments of the original sample to be fractionated, pH 5.0 seems to be the most satisfactory. At pH 4.6 the separation of fractions A and B was not as sharp as at pH 5.0, while at pH 7.0 fraction B (angiotonin) was spread out over a wide area, all of the angiotonin not being recovered even when fifty 10 ml aliquots were collected.

One of the drawbacks of the use of alkaline silver in the purification of angiotonin has been the possible concomitant precipitation of histidine(2). In the present studies, histidine, along with salts and other impurities, was removed in fraction A which was found to give a strong color reaction with Ehrlich's diazo reagent. Edman(6) eliminaed histidine by electrodialysis. It appears that histidine can be easily removed under the conditions of the experiments reported in this paper.

Preparations of various degrees of purity have been run through the column at pH 5.0. One which assayed 130 μg per unit was increased in activity to 30 μg per unit; another assaying 50 μg per unit, after chromatography was equivalent to 27 μg per unit. The latter is the purest material separated by one passage through the column at pH 5.0, and its activity compares favorably with the best preparations made by the laborious technics

4. Shipley, R. E., Helmer, O. M., and Kohlstaedt, K. G., *Am. J. Physiol.*, 1947, v149, 708.

5. Moore, S., and Stein, W. H., *J. Biol. Chem.*, 1948, v176, 367.

6. Edman, P., *Arkiv. f. Kemi, Mineralogi och Geologi*, 1945, v22, 1.

of Plentl and Page(2) using metallic salt precipitations. Furthermore, a very high recovery of the angiotonin activity of the starting material can be obtained.

It has been possible to separate angiotonin in a salt-free condition from saturated sodium chloride solutions and from solutions containing ammonium sulfate.

By means of the column angiotonin has been demonstrated to be present in the blood stream of cats after the injection of renin. In these experiments the blood of the animal was collected directly into alcohol to prevent possible *in vitro* formation of angiotonin.

Discussion. Since each laboratory has its own standard for testing angiotonin activity it is difficult to compare the purity of the preparations described in this paper to that reported by Edman and by Plentl and Page. Edman's angiotonin gave a ninhydrin color reaction whereas ours did not. The activity in terms of weight may not necessarily be a criterion of purity since angiotonin may be a mixture of polypeptides. The latter question will be investigated.

Summary. A method has been described for the purification of angiotonin by means of a paper pulp column. By using an aqueous solution of phenol as a developing medium, the polypeptide (angiotonin) has been separated from free amino acids and salts and possibly from other inactive polypeptides.

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Direct Determinations of Plasma, Cell, and Organ-Blood Volumes in Normal and Hypervolemic Mice. (18003)

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Transplantation studies of X-ray induced ovarian tumors have shown that all animals bearing estrogen - secreting transplanted growths have a marked increase of plasma volume(1,2), while those bearing transplanted masculinizing growths of lutein cell origin have an associated polycythemia(3). In these earlier studies, the blood volume determinations were made with Evans blue (T-1824) and their accuracy, particularly that of the cell volume, is open to doubt. The duration of the correct mixing time is uncertain and there is some disappearance of dye from the blood during the period now commonly believed to be the "mixing" time(4,5). Furthermore, the venous hematocrit differs from the capillary hematocrit and the two may not parallel each other under abnormal conditions. For these reasons the radioisotope technics have been adapted to the direct determination of cell and plasma volumes in mice and procedures were worked out to obtain approximate information of organ volumes. Changes in organ volumes may aid in detecting the primary site of the plasma-volume rise which in turn may give a clue to the site of albumin production.

Comparative studies of red-cell and plasma volumes using ^{32}P-tagged red cells and T-1824, respectively, have been made recently in man and dogs. Nachman and associates (6) found that in man the red-cell volume as measured with ^{32}P-labeled red cells is about 20% less than the values obtained with T-

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4. Lawson, H. C., Overbey, D. T., Moore, J. C., and Shadle, O. W., *Am. J. Physiol.*, 1947, v151, 282.

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TABLE I.
Literature Data on Red Cell Volumes by Different Technics and Relation of Average Body Hematocrit to Venous Hematocrit.

		Red-cell volume		
		^{32}P	^{59}Fe	Avg body hematocrit
Investigator	Species	T-1824	T-1824	Venous hematocrit
Nachman (6)	Man	.80		.88
Gibson (7)	"		.85	.91
Gibson (7)	Dog		.82	.91
Meneely (8)	Man		.80	.89
Reeve (9)	"	.87		.95
Mayerson (10)	"	.99		.915*
Gregerson (11)	"			.96*

* A correction factor for plasma trapped in hematocrit determination.

1824 dye. The discrepancy is, in their opinion, due in part to differences between hematocrit values in peripheral veins and smaller vessels, and in part to the intrinsic error of the centrifuge hematocrit. They estimate that the body hematocrit is on the average 0.88 times that of the venous hematocrit. Earlier data of others(7-11) are summarized in Table I.

Material and methods. The Evans-blue (T-1824 dye) technic for the determination of plasma volumes in mice has already been reported(1,3). The red-cell volumes were obtained by the injection of ^{32}P-labeled homologous erythrocytes according to the method of Hevesy and Zerahn(12) with a modification by Nieset *et al.*(13). Plasma labeled with ^{131}I was prepared by the procedure of Fine and Seligman(14) with the following modification. The unbound inorganic iodide was removed by passing the iodinated plasma repeatedly through an Amberlite IR-400 anion-exchange resin column, thereby eliminating the 48- to 72-hour dialysis. The organ-blood volumes were obtained as follows: The desired substance was injected in volumes of 0.1 to 0.2 ml into the tail vein of the animal under nembutal anesthesia. The blood was withdrawn from the heart in 5 to 6 minutes, the animal sacrificed, and the hematocrit measured by the capillary technic of Parpart and Ballentine(15). An autoscaler with automatic sample changer (Tracerlab Co., Boston, Massachusetts) was used to determine the radioactivity of the ^{32}P samples and a gamma ionization chamber(16) for that of ^{131}I. The ^{131}I activity was measured by inserting the entire organ in the chamber, whereas to measure ^{32}P activity the organ was dissolved in hot concentrated nitric acid and the resulting solution diluted to a known volume. An aliquot was evaporated to dryness in a cup and counted in the autoscaler. Care was taken to avoid any significant self-absorption by taking aliquots with very little total solid content.

Results. Simultaneous determinations of

6. Nachman, H. M., James, G. W., III, Moore, J. W., and Evans, E. I., *J. Clin. Invest.*, 1950, v29, 258.

7. Gibson, J. G., 2nd, Peacock, W. C., Seligman, A. M., and Sack, T., *J. Clin. Invest.*, 1946, v25, 838.

8. Meneely, G. R., Wells, E. B., and Hahn, P. F., *Am. J. Physiol.*, 1947, v148, 531.

9. Reeve, E. B., and Veall, N., *J. Physiol.*, 1949, v108, 12.

10. Mayerson, H. S., Lyons, C., Parsons, W., Nieset, R. T., and Trautman, W. V., Jr., *Am. J. Physiol.*, 1948, v155, 232.

11. Gregerson, M. I., *J. Lab. Clin. Med.*, 1944, v29, 1266.

12. Hevesy, G., and Zerahn, K., *Acta Physiol. Scand.*, 1942, v4, 376.

13. Nieset, R. T., Porter, B., Trautman, W. V., Jr., Bell, R. M., Parson, W., Lyons, C., and Mayerson, H. S., *Am. J. Physiol.*, 1948, v155, 226.

14. Fine, J., and Seligman, A. M., *J. Clin Invest.*, 1944, v23, 720.

15. Parpart, A. K., and Ballentine, R., *Science*, 1943, v98, 545.

16. A. E. C. Isotopes Division Circular A-7, 1949.

TABLE II.
Plasma Volumes in Normal and Hypervolemic Mice Determined Simultaneously with T-1824 Dye and Plasma Labelled with ^{131}I. Derivation of conversion value 1.2.

Procedure	Normal mice					Tumor-bearing mice: Granulosa				Lut.	Ca.
T-1824	5.5	5.5	5.6	5.8	6.3	8.2	8.4	9.5	9.6	6.1	12.9
^{131}I plasma	4.1	4.9	5.4	4.9	5.3	6.9	7.2	8.1	7.5	5.4	10.6
T-1824 / ^{131}I plasma	1.3	1.1	1.0	1.2	1.2	1.2	1.2	1.2	1.3	1.1	1.2

Plasma volumes are given as % body wt. Lut. = Transplantable luteoma, Strain IX(17). Ca = Transplantable carcinoma of ovarian origin (to be published).

TABLE III.
Plasma Volumes in Normal Rabbits Determined with T-1824 Dye and ^{131}I-Labeled Plasma.

Procedure	Females			Males		
	No.	Range % wt	Avg % wt	No.	Range % wt	Avg % wt
T-1824	9	3.8-5.1	4.3	14	3.5-4.8	4.1
^{131}I-plasma	12	3.0-4.9	4.0	9	2.7-4.4	3.8
T-1824/^{131}I			1.08			1.08

Evans-blue and ^{131}I-plasma volumes were made by introducing these two materials in immediate succession intravenously into the mice and rabbits. The results are shown in Tables II and III. These data indicate that the Evans-blue values, calculated on the basis of dye concentration after the conventional mixing time of 5 to 6 minutes, are higher than the iodinated plasma values. It is noteworthy that the concentration of ^{131}I plasma does not drop significantly within 2 to 5 minutes after injection as does Evans blue, indicating that during the so-called mixing time the dye is lost from the circulation. No difference was found in the values when either Evans blue or ^{131}I plasma was injected first.

Simultaneous direct cell and plasma volume determinations were made by the injection of ^{32}P-tagged homologous cells and Evans blue. The results in normal and in tumor-bearing mice are shown in Table IV. The Evans-blue values were divided by a factor of 1.2 to obtain the presumably correct plasma-volume concentration. These data indicate that the cell volumes, as calculated by the conventional Evans blue-hematocrit technic, are above those obtained by direct cell determinations by a factor of 1.54. Since the disappearance curves indicate thorough mixing of both plasma and cells at time of sampling it follows that blood in smaller vessels contains a smaller percent of cells than in the large vessels.

The direct determination of plasma and cell volumes enables a calculation of the average body hematocrit. This ratio of average body hematocrit to the venous hematocrit is 0.88 (Table IV). Thus all data indicate that there is a greater concentration of plasma in capillary than in venous blood by an unknown factor which is smaller than 0.88.

Data on three types of neoplasms are presented in Table IV: (a) an estrogenizing granulosa tumor, (b) a masculinizing luteoma, and (c) ovarian carcinoma causing no secondary changes in sex organs. The granulosa tumor causes a marked plasma-volume rise without a drop in red-cell volume, the luteoma a marked rise in cell volume with a slight but distinct rise in plasma volume and the ovarian carcinoma a moderate plasma-volume rise. These observations confirm those made earlier with T-1824(1) although

17. Furth, J., and Sobel, H., *Cancer Res.*, 1947, v7, 246.

TABLE IV.
Simultaneous Red-Cell and Plasma-Volume Determination in Mice.

Mice	No. in group	Red cell vol.			Plasma vol.		Total blood vol.			Hematocrit		
		^{32}P	T-1824	T-1824 ^{32}P	^{32}P	T-1824 1.2	^{32}P	T-1824	^{32}P + T-1824 1.2	Ven.	Avg body	Avg Ven.
Normal Rf/Ak ♂	3	3.31	5.10	1.54	4.20	5.14	7.52	11.27	8.45	44.2	39.2	.89
" " ♀	2	3.00	5.03	1.68	4.18	5.51	7.18	11.64	8.51	42.1	35.2	.84
Normal Rf ♂*	6	2.66	3.92	1.47	3.74	4.54	6.39	9.36	7.20	41.5	37.0	.89
Granulosa ♂ †	2	3.55	4.93	1.39	9.00	10.34	12.55	17.35	13.89	28.2	25.6	.91
" ♀ †	3	3.45	5.80	1.68	5.75	8.17	9.21	15.59	11.62	37.6	30.2	.80
Ov. carcinoma ♂ †	2	2.80	3.67	1.31	5.93	7.47	8.74	12.63	10.28	29.0	27.2	.94
Luteoma ♂ †	2	4.35	7.25	1.67	4.23	5.65	8.58	14.04	10.0	50.5	43.3	.86
Avg				1.54								.88

The figures represent averages. *These males were fighting in the cage and may have been dehydrated. † Tumor-bearing mice.

the values published require corrections as indicated in Fig. 1.

The organ-blood volume values (Table V) are given here chiefly to indicate the usefulness of this procedure in such studies as hypervolemia, polycythemia and shock. These determinations yielded unexpectedly low blood-volume values in tumors. In sections, the tumors are rich in giant thin-walled vessels and, on the basis of fixed and stained microscopic sections, the tumors would appear to have a relatively high blood volume. It is possible that the circulation in tumors is sluggish; if so, tumor cells live in a state of relative anoxia. Experiments are in progress to ascertain the facts concerning circulation in tumors in order to determine the correctness of this assumption.

Discussion. Iodinated homologous plasma circulates in the blood for a period of 10 to 20 minutes at close to the level reached within a minute or two after injection and falls very slowly thereafter, whereas the Evans-blue values drop rapidly for 1 to 5 minutes following injection. Therefore, it can be presumed that this disappearance of Evans blue is due, not to mixing, but to withdrawal of dye from the circulation during this period. Evans blue like ^{131}I circulates in the blood in a form bound to blood proteins. The recent studies of Kruse and McMaster(18) indicate that protein-bound Evans blue is taken up primarily by reticuloendothelial cells (macrophages). The rapid withdrawal from the blood of various materials by the macrophage system parallels the rapid drop of Evans-blue tagged protein. Thus it is probable that Evans blue is actually well mixed within a minute or two, that some dye is withdrawn during this mixing period and that its subsequent drop is due to withdrawal mainly by macrophages. However, postmortem examinations of animals at a later time indicate widespread blue discoloration of organs that are not part of this system. The possible use of the rate of disappearance of Evans blue from the blood as a measure of reticuloendothelial function requires further study. In

18. Kruse, H., and McMaster, P. D., *J. Exp Med.*, 1949, v90, 425.

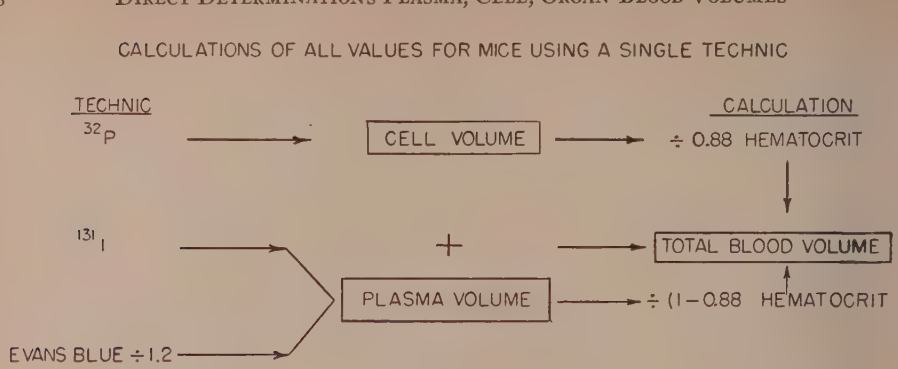

FIG. 1.

TABLE V.
Organ-Blood Volumes by the ^{131}I-Tagged Plasma Technic.

Mice	No. in group	Pl. vol. % body wt	Plasma volume (ml) per g of tissue				
			Spleen	Lung	Kidney	Liver	Tumor
Normal	9	5.1	.11	.23	.16	.20	—
Tumor bearing:							
Granulosa	5	7.5	.11	.30	.21	.24	.03
Ovarian ca.	2	8.5	.09	.22	.28	.35	.01
Luteoma	1	5.4	.06	.24	.19	.23	.02

The figures are averages. Most tumors in this series were of small or medium size.

both normal and hypervolemic mice there was a ratio of approximately 1.2 between Evans-blue and ^{131}I-plasma values. Thus the Evans-blue determinations are useful and nearly accurate if such a correction value is applied. It deserves emphasis that blood volumes calculated with the aid of the conversion values here given are only approximate and exact values can be obtained only by direct determinations. The conversion figures are based on estimated average body hematocrits; but the capillary hematocrits are expected to vary in different physiological and pathological states introducing an error of unknown magnitude.

Summary and conclusion. Direct single and combined cell- and plasma-volume determinations were made on normal and polycythemic mice with ^{32}P-tagged red cells and ^{131}I-tagged plasma. The data indicate that: (a) The Evans-blue plasma volumes in mice are above the probably more correct ^{131}I-plasma volumes by a factor of 1.2 and in rabbits by a factor of 1.08. (b) The so-called mixing time of Evans blue is a withdrawal time of this dye by Evans blue-affin cells, most of which are probably macrophages. (c) The cell volume calculations based on Evans-blue values are higher than those obtained by direct determinations with the ^{32}P technic by a factor of 1.56 in normal and 1.51 in tumor-bearing mice tested. (d) The average body hematocrit in mice is approximately 0.88 times the venous hematocrit. (e) On the basis of these conversion values a calculation of all values can be made if only one technic is employed (Fig. 1). (f) Hypervolemia caused by estrogen-secreting tumors is attributable to an almost selective plasma-volume rise while that with luteoma is due primarily to polycythemia and, to a lesser extent, to a plasma volume rise.

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Potentiation of Androgen by Aqueous Aluminum Phosphate Suspension. (18004)

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Carlinfanti, D'Alo, and Cutolo(1,2) applied to testosterone, with striking results, the Holt(3) aluminum phosphate suspension method used in the preparation of water-soluble diphtheria toxoid. Their data indicated that a depot effect also occurs when small quantities of aluminum phosphate are added to water-insoluble testosterone. Characteristic of the depot effect of aqueous testosterone-aluminum phosphate suspensions was a 2- to 3-fold potentiation, and increase in duration of activity. As an outcome of the reports by these authors, experiments were undertaken in an attempt (1) to confirm the work of Carlinfanti and his associates in the guinea pig; (2) to extend the data to another species, the albino rat.

Immature male guinea pigs obtained from Manor Farms and immature Sprague-Dawley male rats were castrated, and immediately injected with a single dose of the various androgen preparations. In order to study the degree and duration of androgenic stimulation, groups of animals were sacrificed and examined at predetermined intervals. In both species, growth response of the seminal vesicles was the end-point in evaluating potency. The androgen preparations assayed were (1) an aqueous testosterone-aluminum phosphate (ATAP) suspension prepared according to the method described by Carlinfanti *et al.*, (2) an aqueous testosterone suspension, and (3) testosterone propionate oil solution. Each preparation contained 25 mg/cc of testosterone or testosterone propionate, and was administered on a body weight basis.

Exp. 1. Guinea pigs weighing 200 to 300 g were injected after castration with a single subcutaneous dose of 25 mg of androgen per 100 g of body weight under the skin of the back. Groups (3 to 5 guinea pigs per preparation) were autopsied at days 3, 6, 13, 23, 31, and 35 after injection. Body weights were recorded; seminal vesicle, adrenal, liver, kidney, and pituitary weights also were taken, and sections of these organs were prepared for histological examination. Table I summarizes and Fig. 1 illustrates the outcome of this experiment. The values closely resemble those reported by Carlinfanti for the guinea pig. Examination of the time response curves obtained by Carlinfanti and by us in the guinea pig suggests that the effect of ATAP suspension lasts at least twice as long as that of testosterone propionate oil solution.

Exp. 2. Immature rats, 27 days old, were castrated and injected intramuscularly with a single dose of 25 mg of androgen per 100 g of body weight. Groups (4 rats per preparation) were killed on days 3, 7, 14, 24, and 32. At autopsy, body weights were taken. The seminal vesicles, ventral prostates, adrenals, livers, and kidneys were weighed and portions of these organs preserved for histological examination. The data obtained with rats show that $AlPO_4$ in aqueous suspension potentiates the androgenic effect of testosterone (Fig. 2). Seminal vesicle response to aqueous testosterone suspension with aluminum phosphate was more than twice that found for aqueous suspensions containing testosterone alone. Maximum effect was obtained 14 days after injection as compared with a maximum at 7 days for aqueous testosterone suspension. Approximately 20 days after injection of ATAP suspension, the seminal vesicle weight still equaled the maximum obtained with an aqueous testosterone suspension on day 7. Apparently a 2- to 3-fold increase in duration of action occurred.

Exp. 3. As a further test of the effectiveness of the new preparation, vials of a commercially available aqueous testosterone sus-

1. Carlinfanti, E., D'Alo, F., and Cutolo, L., *Riv. ist. sieroterap. ital.*, 1948, v23, 151; Abstr. *Chem. Abst.*, 1949, v43, 3085.

2. Carlinfanti, E., D'Alo, F., and Cutolo, L., *Lancet*, 1949, v1, 479.

3. Holt, L. B., *Lancet*, 1947, v1, 282.

TABLE I.
Androgenic Response of Immature Castrate Guinea Pigs to Aqueous Testosterone Aluminum Phosphate Suspension.

	Carlinfanti	Schering
Onset of potentiation	10-15 days	13 days
Max. response	30 "	31 "
Potency ratios:		
Testosterone $AlPO_4$ vs. testosterone propionate in oil	1.9	2.9
Testosterone $AlPO_4$ vs. controls	5.8	8.3
Testosterone propionate in oil vs. controls	3.1	2.8

pension were purchased and divided into 2 lots. One lot was treated with aluminum phosphate using a modified Carlinfanti suspension method; the other remained unaltered. Again, there was a significant potentiation and prolongation of androgenic activity with the aluminum phosphate-treated product when compared with the commercial preparation containing no aluminum phosphate (see Fig. 3).

It has been demonstrated that esters of testosterone with short-chain fatty acids elicit a greater biological response than free testosterone when administered in oil (Miescher, Wettstein, and Tschopp)(4). Kochakian(5) and others have found enhanced biological response to testosterone in experimental animals when given as an aqueous suspension. If the increase in activity of testosterone esters in oil over that of free testosterone in oil depends merely on

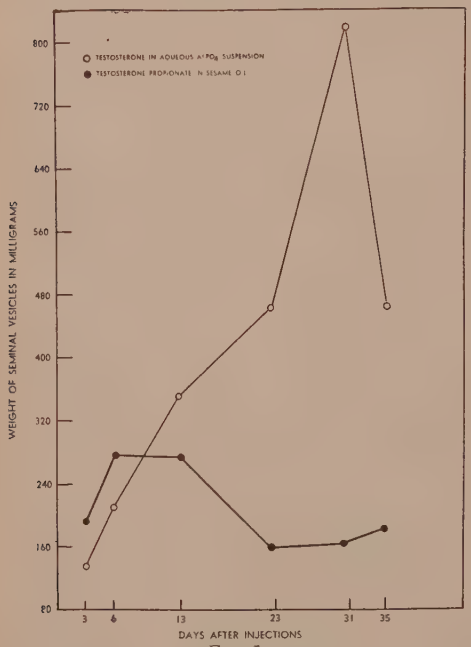

FIG. 1.
The depot effect of ATAP suspension. Seminal vesicle response in immature guinea pigs to a single subcutaneous injection of androgen.

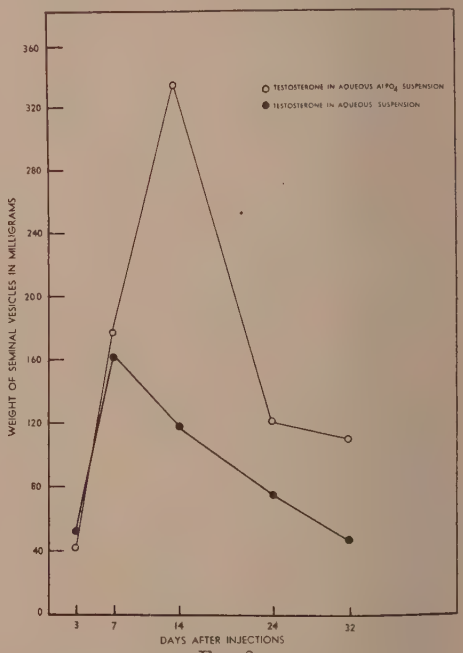

FIG. 2.
The depot action of ATAP suspension. Seminal vesicle response in immature castrate rats to a single intramuscular injection of testosterone.

4. Miescher, K., Wettstein, A., and Tschopp, E., *Biochem. J.*, 1936, v30, 1977.

5. Kochakian, C. D., *Endocrinology*, 1938, v22, 181.

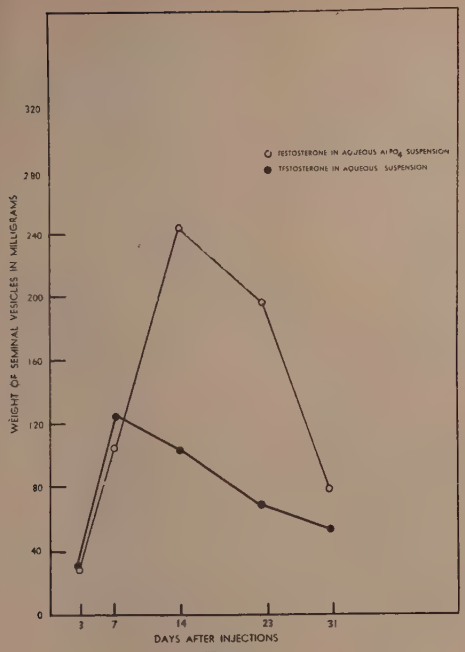

FIG. 3.

Potentiation of a commercial aqueous testosterone suspension by aluminum phosphate. Seminal vesicle response of immature rats to a single intramuscular injection.

slower release from the depot site, it follows that a depot of micro-crystalline testosterone, subcutaneously or intramuscularly, should be ideal for slow absorption. As yet, there is no experimental demonstration of the mechanism by which aqueous $AlPO_4$ suspension of testosterone elicits a greater androgenic response than the aqueous suspensions without $AlPO_4$.

Summary. The experimental data confirm the depot effect described by Carlinfanti and his co-workers following subcutaneous injection of ATAP suspensions in the guinea pig. The effect also has been demonstrated in immature castrate rats after intramuscular injection. In both instances marked increase in response and duration of biological activity were obtained. Potency ratios and indices of duration of action indicate that each milligram of testosterone in aqueous aluminum phosphate suspension is (1) two or more times as active, and (2) acts approximately two to three times as long as the same quantity of testosterone in aqueous suspension alone.

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Inhibition of the Phagocytic Action of Leucocytes by Mumps and Influenza Viruses. (18005)

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In previous experiments(1-3), it has been shown that mumps virus has a destructive effect on the erythrocytes from a number of species which results in hemolysis. It was therefore of interest to determine if this virus would damage other cells. Polymorphonuclear leucocytes of the guinea pig were selected for study since their ability to engulf killed bacteria offered a ready test of their intact functional activity for which a quantitative technic has been developed for direct observation with the microscope(4). The erythrocytes of this species are very susceptible to the hemolytic action of mumps virus (3).

The effect of influenza virus on the phagocytosis of bacteria was also studied since reports(5) indicated that this virus caused

1. Morgan, H. R., Enders, J. F., and Wagley, P. F., *J. Exp. Med.*, 1948, v88, 503.

2. Chu, L. W., and Morgan, H. R., *J. Exp. Med.*, 1950, v91, 393.

3. Chu, L. W., and Morgan, H. R., *J. Exp. Med.*, 1950, v91, 403.

4. Merchant, D. J., *J. Inf. Dis.*, in press.

agglutination of guinea pig leucocytes. However, influenza virus did not possess the hemolytic property demonstrated by mumps virus(1).

Materials and methods. The egg-adapted strain of mumps virus described in previous studies was employed(1,2). The virus was cultivated in the amniotic sac of 7-8-day-old chick embryos harvesting the amniotic and allantoic fluids after incubation at 35°C for 5 days. The PR8 strain of influenza A virus was inoculated into the allantoic cavity of 10-day-old embryos and the allantoic fluid was collected after 48 hours incubation at 35°C. All of these virus-containing egg fluids were dialyzed against saline buffered with phosphate at 4°C for 48 hours before use in these experiments. The mumps antiserum employed was prepared by immunizing guinea pigs.

Estimations of the phagocytic activity of the polymorphonuclear leucocytes of the guinea pig were carried out by the technic recently described in detail using killed suspensions of *Bacillus anthracis* as the test organism(4). The phagocytosis of these bacteria was observed directly in a slide preparation on a warm stage using darkfield illumination. The mixture placed on the slide for observation consisted of 0.7 ml of homologous serum, 0.1 ml of a leucocyte suspension containing 45,000 to 50,000 cells per cmm and 0.1 ml of a suspension of anthrax bacilli. The suspension of anthrax bacilli was added after 15 minutes of incubation at 37°C. In the test experiments, 0.1 ml of the dialyzed virus preparation was added, and, in the inhibition experiments, 0.1 ml of the immune serum was added to 0.6 ml of the normal guinea pig serum. Phagocytosis was evaluated after 15 minutes at 37°C by determining the percentage of cells that had engulfed the anthrax organisms.

Results. Table I presents a summary of the results of representative experiments. In the presence of mumps or influenza viruses, the ability of guinea pig polymorphonuclear leucocytes to engulf anthrax bacilli was reduced 20-30%. Amniotic or allantoic fluid containing mumps virus had identical effects. Heating of either virus at 50°C for 40 minutes destroyed this inhibitory action which was also specifically neutralized by the addition of homologous immune serum in the case of mumps virus. Amniotic or allantoic fluids harvested from normal embryonated eggs had no effect on the phagocytic activity of the leucocytes.

TABLE I.

Effects of Mumps and Influenza Viruses on Phagocytic Activity of the Polymorphonuclear Leucocytes of the Guinea Pig.

Materials added	Phagocytosis, %
Mumps virus	42
" " heated at 50°C, 40 min.	76
Mumps virus + mumps antiserum	70
Control	68
PR8 influenza virus	49
" " " heated at 50°C. 40 min.	81
PR8 influenza virus + mumps antiserum	48
Control	75

Another effect was observed after the addition of these viruses. Using an ocular micrometer, the diameter of the leucocytes was measured and found to be reduced 10-15%. This is a rough measure of their ability to spread out at a liquid-solid interface. This effect disappeared when the virus was heated or immune serum was added. No morphological changes were observed inside these cells.

Discussion. Evidence presented in these experiments indicates that mumps virus has an action on guinea pig leucocytes which decreases their phagocytosis of bacteria. The specific nature of this activity is indicated by its neutralization by homologous immune serum and the failure of normal egg fluids to exert any similar effect. This property of the virus is similar to its hemolytic activity in its susceptibility to inactivation by heat under conditions which leave the hemagglutinating capacity of the virus intact. It would not seem likely however that the action on leucocytes and erythrocytes are different manifestations of a single property of mumps virus since influenza virus, which has no

5. Nungester, W. J., Gordon, J., and Collins, K., *J. Inf. Dis.*, in press.

hemolytic activity, also inhibits phagocytosis. Unless, of course, the inhibition of phagocytosis produced by mumps virus is due to some property entirely different from that of influenza virus causing a similar inhibition. This question awaits further elucidation. One obvious implication of these studies is the possible relationship of inhibition of phagocytosis by these viruses to the development of secondary bacterial infections during the course of diseases caused by them. The significance of these findings must await parallel demonstration of such activity for those viruses in natural or experimental infections before any such conclusions can be drawn.

Summary. Mumps virus and influenza A virus (PR8) have been shown to reduce the phagocytosis of bacteria by the polymorphonuclear leucocytes of the guinea pig *in vitro*. This inhibitory effect is destroyed by mild heating of the viruses which leaves their hemagglutinating capacity intact. Mumps antiserum prevents the inhibition of phagocytosis by mumps virus but has no effect on the inhibitory action of influenza virus.

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A Note on Electrophoresis in Hypothyroidism.* (18006)

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A number of investigators have reported a moderate elevation of total protein in hypothyroidism and have attributed this to hemoconcentration(1-4). With adequate thyroid therapy the total protein concentrations returned to normal. Lewis, McCullagh and Clark(3) included six cases of hypothyroidism in their electrophoretic analyses of the plasma proteins in thyroid disease, and have described a pattern which they consider consistent. These authors worked with a phosphate buffer at pH 7.8 which was the condition under which sera had been studied at that time. However, at the present the general practice is to work in a veronal buffer of pH 8.6 and we have studied several cases under these conditions. Our results differed somewhat from those reported by Lewis *et al.*, and the differences probably are due to this variance of conditions.

Material and methods. The material was obtained from 3 females with spontaneous myxedema and 1 male with post-operative myxedema. In 2 of these patients samples were taken after treatment with desiccated thyroid. One of these latter cases was unable to tolerate adequate doses of thyroid (patient P) because of progressive heart disease. No attempt was made to differentiate pituitary from spontaneous hypothyroidism. The case histories of the patients follow: Case R, a 65-year-old white female housewife, noticed apathy, weakness and dryness of the skin one year prior to admission. Her physical appearance was typical of advanced myxedema *viz.* coarse dry skin, slow hoarse voice, puffy eyelids and absent axillary and pubic hair. The essential laboratory findings were BMR-21, EKG rate 60 with low voltage and flattened T waves, X-ray chest—diffuse

* The authors wish to express their thanks to Dr. Emanuel J. Feinhandler for permission to study his patient.

1. Gildea, E. F., Man, E. B., and Peters, J. P., *J. Clin. Invest.*, 1939, v18, 739.

2. Shirer, J. W., *Trans. Am. Assn. Study Goiter*, 1932, p. 89.

3. Lewis, L. A., McCullagh, E. P., and Clark, J., *Am. J. Med. Sci.*, 1944, v208, 727

4. Moore, D. H., Levin, L., and Smelser, G. K., *J. Biol. Chem.*, 1945, v157, 723.

cardiac enlargement, cholesterol ranged from 820 to 1000 mg % in the serum with the esters comprising 60 to 75% of the total. RBC 2,900,000, WBC 5,200. A remarkable improvement was noted on thyroid extract up to grains II daily with complete amelioration of symptoms. The EKG, blood picture and cardiac configuration returned to normal limits.

Case P, a 64-year-old white female housewife had symptoms of coronary insufficiency with episodes of acute left ventricular failure for 4 years prior to admission. She presented typical myxedematous facies, voice and personality. The BMR was –29, the total serum cholesterol 595 mg % with 450 mg % esters, the RBC 2,780,000, the WBC 5,900 and the hemoglobin 9.3%. The EKG pattern was compatible with hypothyroidism in addition to left ventricular strain. The heart on X-ray configuration was markedly enlarged to right and left. She was unable to tolerate more than one grain of thyroid daily without further anginal symptoms and cardiac embarrassment. She suffered an acute myocardial infarction and expired 6 months after beginning therapy with thyroid.

Case O, a 70-year-old white female housewife complained of symptoms suggestive and diagnosed as coronary insufficiency for 12 years prior to admission to the hospital. She also complained of weakness and severe pain and stiffness of all joints. Her appearance and manner were classical for advanced myxedema *viz.* wax pallor and coarseness of skin, puffy eyelids, slow hoarse voice, and absent pubic and axillary hair. The BMR was –30, the EKG revealed a rate of 58 with flattened T waves. The serum cholesterol was 510 and the cholesterol esters 400 mg %. The RBC was 2,700,000, WBC 4,600 and hemoglobin 10%. A dramatic complete recovery was effected on thyroid extract up to grains II daily with complete cessation of all symptoms including those of coronary insufficiency. She was converted from a vegetative existence to a normal life.

Case S, a 72-year-old retired male tailor underwent a thyroidectomy for symptoms of hyperthyroidism in 1934, and subsequently developed symptoms and signs of thyroid deficiency *viz.* weakness, apathy, dry, coarse skin, severe constipation, absent pubic and axillary hair and a typical myxedematous appearance and manner. The BMR was –32, the EKG revealed a rate of 40 with normal T waves and voltage. The roentgenogram of the heart showed a moderate left ventricular hypertrophy. The serum cholesterol was 310, the esters 215, the RBC 3,560,000, WBC 5,900 and hemoglobin 11%. His response to thyroid up to grains III daily was only fair, but he continued to live a normal life.

The sera were prepared and stored in the frozen state for 1 to 3 days. Five to 6 ml of sera were diluted with 2 volumes of veronal buffer (N/10 and pH 8.5 to 8.6) and dialysed for 24-48 hours at 2°C against 2 liters of buffer. The electrophoresis was carried out in the Tiselius apparatus as modified by Longsworth(5). The field strength was about 5.8 volts cm^{-1}, the time about 14000 sec and the temperature 1.2°C. The patterns were magnified 2.5 to 3.0 times and the relative protein concentrations determined on the descending patterns according to Tiselius and Kabat(6). The chemical A/G ratios were determined by the Howe procedure using the quantitative biuret method for protein determination.

Results and discussion. The results are summarized in Table I. The albumin fractions were low to a varying degree. This is partly in agreement with the findings of Lewis, *et al.* In case R, the administration of thyroid caused a satisfactory clinical response and the albumin rose to the normal range. In case P, the patient was unable to tolerate a therapeutic dose *(vide supra)* and the albumin remained quite low. Although the clinical findings in the other 2 cases, O and S, indicated hypothyroidism of a severity equal to that of the other 2 cases, the albumin values were only slightly depressed. It would seem, therefore, that a low albumin is not an invariable finding in hypothyroidism. Alpha-1 globulin was normal or very slightly

5. Longsworth, L. G., *Chem. Rev.*, 1942, v30, 323.

6. Tiselius, A., and Kabat, E. A., *J. Exp. Med.*, 1939, v69, 119.

TABLE I.
Clinical and Chemical Data on Patients.

Patient	Total protein	Alb., %	Alpha 1, %	Alpha 2, %	Beta, %	Gamma, %	A/G	
							Electrophoretic	Chemical
R1	7.1	49.0	3.7	12.4	17.6	17.5	0.96	1.84
R2*	6.7	57.4	3.5	10.3	16.1	12.6	1.31	—
P1	7.3	41.9	4.5	12.1	19.1	22.2	0.72	1.15
P2*	9.1	39.0	6.2	15.6	11.9	27.2	0.64	—
O	8.0	57.5	3.2	14.8	11.9	13.5	1.36	2.48
S	7.0	55.5	2.5	8.5	18.8	15.1	1.23	1.69
Normal†		63 ±3	5 ±1	7 ±1	13 ±2	12 ±3		

*Following treatment.
† Moore, Dan H., *J. Biol. Chem.*, 1945, v161, 21.

lowered. Under the conditions of their experiments Lewis *et al.* reported no alpha-1 component. The alpha-2 component in 3 cases of "spontaneous" hypothyroidism were high. In the fourth case, the value was normal and, it should be noted, the hypothyroidism resulted after thyroidectomy. This is in marked contrast to the low values for alpha globulin reported by Lewis and co-workers. These authors, however, used a phosphate buffer of pH 7.8 and under these conditions the alpha component is the same as our alpha-2 component. Comparing the values for alpha-2 globulin before and after treatment with thyroid it is seen that in the patient who responded to treatment (R) the alpha-2 component returned to normal whereas in the patient that did not tolerate adequate treatment the value continued to rise.

In 3 out of 4 cases the beta globulin fraction was elevated and in the fourth case (S) the value was normal. Lewis *et al.* reported high values for this component also. Since Beta globulin fraction is generally believed to consist of lipoprotein complexes, and hypercholesterolemic and hyperlipemic conditions have been reported to give increases in this fraction(7). In view of this possible correlation, it was felt desirable to compare the beta globulin values with the total cholesterol values. Case O had a high cholesterol and a normal beta globulin. On the other hand, in case S, the beta globulin was very high and the total cholesterol was only slightly elevated. Case R, with a total cholesterol of 1000 mg % had a beta globulin fraction of 17.6%, whereas in case P with a lower total cholesterol (595 mg %) the beta globulin fraction was higher (19.1%). It appears, therefore, that in our cases there was no consistent correlation between beta globulin and cholesterol. In view of the results of Kunkel and Ahrens(7), a study of other blood lipids would have been of value but was not done.

The gamma globulin values in 2 cases were elevated and in the other 2 were normal. This is in agreement with the general findings of Lewis *et al.* As in the case of all the other components, the high value of gamma globulin before treatment changed to a normal value following treatment. It is worthy of note that the one case which could not be adequately treated (P) the gamma globulin showed a continued rise to extremely high values. This also happened in a terminal case of Gaucher's disease which we studied, where the gamma globulin rose to a value of about 40% of the total protein 3 days before death.

In conclusion, we feel that no characteristic electrophoretic pattern can be assigned to hypothyroid disease in general on the basis of available data.

Summary. The electrophoretic patterns of the sera of 4 hypothyroid patients were

7. Kunkel, H. G., and Ahrens, E. H., Jr., *J. Clin. Invest.*, 1949, v28, 1565.

8. Longsworth, L. G., Shedlovsky, T., and MacInnes, D. A., *J. Exp. Med.*, 1939, v70, 399.

studied, 2 before and after thyroid administration. The results have been compared with those of other authors. On the basis of all the available data there does not appear to be a characteristic distribution of protein fractions in hypothyroid disease.

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